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THE INFLUENCE OF QUINONES ON THE OVARIAN RESPONSE OF IMMATURE RATS TO GONADOTROPIC STIMULATION[1]

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ABSTRACT

A series of quinones and napthoquinones have been tested in immature female rats for their ability to inhibit gonadotropins. Hydroquinone, 2,6-dimethylhydroquinone and 2-methyl-napthoquinone produced no significant inhibition of the action of pregnant mare serum gonadotropin (PMS). Trimethylquinone and lapachol were effective in modifying the response of the ovary to PMS. The inhibition of a given dosage of PMS was proportional to the concentration of lapachol administered. Lapachol clearly depresses the ability of the ovary to respond at all levels of PMS, and exerts a marked inhibitory action even when the animal is given excessively large doses of the gonadotropin. The percentage inhibition produced by a given dosage of lapachol appears to be constant over the range of PMS concentrations that were tested (5–100 I.U.). In contrast, lapachol was unable to inhibit the action of chorionic gonadotropin (PU) or crude extracts of rat pituitaries. Neither hydrolapachol nor lomatiol was an effective inhibitor of PMS. There was a reduction in the succinoxidase activity of ovarian homogenates from both PU and PMS treated rats injected with lapachol. The manner in which quinones inhibit the ovarian response to PMS is discussed.

DURING the last decade the quinones and their reduction products, the hydroquinones, have received considerable attention in the fields of antimalarial (1) and antibiotic (2, 3) pharmacology. The simple quinones have been reported to be highly effective mitotic poisons that block mitosis at the metaphase (4, 5). Moreover, Ball, *et al.* (6) have demonstrated that the more complex napthoquinones can selectively poison

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respiration by blocking the link between succinic dehydrogenase and the cytochrome system.

Sanyal (7) has suggested that the substance responsible for the sterility effect of peas in rats is 2,6-dimethylhydroquinone. Parenteral administration of 2,6-dimethylhydroquinine or hydroquinone produced a transient inhibition of the estrous cycle of rats, whereas other quinones were inactive (8). Noble and Graham (9) have found that many quinones can mimic *Lithospermum ruderale* by inhibiting the responses of the immature rat ovary to the gonadotropins of pregnant mare serum (PMS).

In consideration of the known ability of napthoquinones to act as respiratory poisons, an investigation was launched to compare the efficacies of the most promising compounds for the inhibition of the ovarian response to gonadotropins.[4]

METHODS

Immature female rats (Harvard strain), 22 to 23 days old and weighing 50–65 gms., were used throughout these experiments. The quinones, in aqueous medium, were administered subcutaneously twice daily for four days. On the second day of quinone treatment, each rat was given a single injection of PMS.[5] Animals treated with chorionic gonadotropin (PU)[6] were given three daily injections beginning on the second day of quinone treatment. The subcutaneous injections of the hormones and quinones were made at different sites to prevent mixing. Control groups received concentrations of PMS and PU corresponding to their respective experimental groups.

The quinones were prepared for injection as follows: hydroquinone and 2,6-dimethylhydroquinone were dissolved in 0.9% saline; 2-methylnaphthoquinone was suspended in sesame oil; trimethylquinone, 2,3,5-trimethylquinone-6-β-thioacetic acid and 2,3,5-trimethylquinone-6-β-thiopropionic acid were dissolved in propylene glycol; 2-hydroxy-3-(3-methyl-2-butenyl) napthoquinone (lapachol), 2-hydroxy-3-(3-methyl-2-lbutanyl) napthoquinone (hydrolapachol) and 2-hydroxy-3-(3-hydroxy-methyl-2-buteny)napthoquinone (lomatiol) were suspended in propylene glycol by heating until solution was complete and then cooling rapidly.

Seventy-two hours after the initiation of hormone administration the animals were sacrificed and the ovaries and uteri were removed and weighed. The uterine weights included the fluid that may have been contained in the lumen. Ovaries were fixed in Bouins solution for histological examination.

Two 1.0 ml. aliquots of a three per cent ovarian homogenate were used in the assay of succinoxidase activity by the method of Schneider and Potter (10). Total nitrogen was determined in aliquots of the homogenate by nesslerization after digestion in a selenium-sulfuric acid mixture.

RESULTS

Pregnant mare serum contains a gonadotropin that stimulated predominantly follicular development, and in the immature rats used in these

[4] A preliminary report of this work was presented at the annual meeting of the American Society for Pharmacology and Experimental Therapeutics, November, 1950 (13).

[5] "Gonadogen" (Upjohn).

[6] "Antuitrin S" (Parke-Davis).

experiments it was found that 2 I.U. would produce estrogen secretion as indicated by increase in uterine weight, without perceptible increase in ovarian weight. Doses of PMS above 2 I.U. brought about increases in ovarian weight approximately in proportion to the dose until a maximum was reached at 40 I.U. Larger doses may result in somewhat smaller ovaries that show more extensive luteinization.

Hydroquinone, 2,6-dimethylhydroquinone and 2-methylnapthoquinone produced no significant inhibition of PMS action (Table 1). Trimethyl-

TABLE 1. EFFECT OF QUINONES ON THE RESPONSE OF THE OVARY OF IMMATURE RATS TO PMS STIMULATION

Agent	PMS* (40 I.U.)	Quinone dosage (mg./day/4 days)	Rats	Ovarian wt. (mg.)†	Uterine wet wt. (mg.)†
None	−	—	40	18.2 ± 1.3	22.4 ± 1.5
None	+	—	60	152.6 ± 5.2	105.1 ± 3.7
Hydroquinone	+	0.4	6	152.9 ± 6.8	103.0 ± 4.8
2,6-dimethyl-hydroquinone	+	2.0	6	143.0 ± 8.2	—
2-methylnapthoquinone	+	0.5	6	161.2 ± 12.6	—
Trimethylquinone	+	0.5	10	114.3 ± 5.7	101.5 ± 2.7
		1.0	8	99.1 ± 5.4	100.4 ± 4.7
2,3,5-Trimethylquinone-6-β-thiopropionic acid	+	4.0	6	112.5 ± 4.0	105.0 ± 8.8
2,3,5-Trimethylquinone-6-β-thioacetic acid	+	4.0	8	139.5 ± 8.5	108.0 ± 6.0
Lapachol (2-hydroxy-3-(3-methyl-2-butenyl)napthoquinone)	+	2.0	10	135.3 ± 9.7	104.0 ± 3.4
		6.0	10	112.1 ± 4.5	95.1 ± 3.0
Hydrolapachol (2-hydroxy-3-(3-methyl-2-butanyl)napthoquinone	+	6.0	12	151.5 ± 10.8	105.0 ± 4.5
		12.0	12	150.7 ± 10.3	101.2 ± 4.2
Lomatiol (2-hydroxy-3-(3-hydroxymethyl-2-butenyl)napthoquinone	+	6.0	8	165.2 ± 9.0	90.3 ± 4.6

* On the second day of quinone treatment each rat received 40 I.U. PMS
† Mean ± S.E.

quinone and lapachol, however, were effective in modifying the response of the ovary. Trimethylquinone was effective at a lower dosage than lapachol, but it was also highly toxic. In toxicity studies, the LD_{50} of lapachol was found to be 16 mg. daily per rat for four days while that for trimethylquinone was 1.5 mg. daily when given for the same period.

The thiopropionic acid derivative of trimethylquinone was a less effective inhibitor of PMS than the parent compound, and the introduction of the side chain also reduced the toxicity of the substance. The thioacetic acid derivative was an exceedingly poor inhibitor of PMS. Neither hydrolapachol nor lomatiol were effective in inhibiting the ovarian response (Table 1).

The values recorded in Figure 1 show that the inhibition of the ovarian response to 40 I.U. of PMS was proportional to the amount of lapachol ad-

ministered. In contrast, lapachol was unable at any dosage to inhibit the action of PU (Fig. 1). Although only two dose levels of trimethylquinone could be employed (Table 1), the indications were that this inhibition was also not an all or none response, but rather that the amount of depression was related to dose. This was also true of the thioacetic and thiopropionic acid derivatives of trimethylquinone. At similar concentrations these compounds were unable to inhibit the action of PU.

Lapachol (6 and 12 mg./day for 4 days) was unable to inhibit the ovarian response in animals treated with a crude extract of rat pituitaries. The

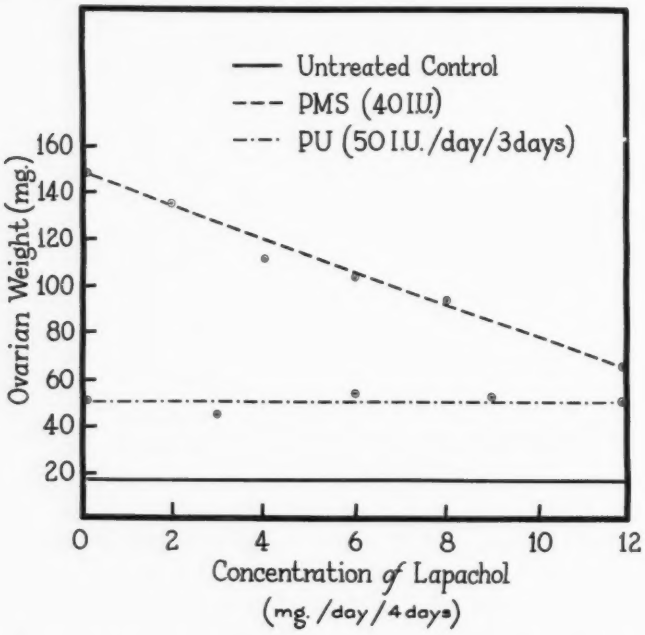

FIG. 1. The effect of lapachol on the ovarian response to gonadotropic stimulation.

adrenal response to ACTH (20 I.U./day for 3 days) was also unaffected by large doses of lapachol, as was the estrous cycle of rats receiving a daily dose of 6 or 20 mg. for a period of three weeks (11).

According to the evidence presented in Table 2, lapachol clearly depresses the ability of the ovary to respond at all levels of PMS and exerts a marked inhibitory action even when very large doses of the gonadotropin are given. The percentage of inhibition appears to be fairly constant over the range of PMS concentrations tested.

Striking changes were noted in the histological appearance of the ovaries. The inhibitory effect of lapachol on the gonadotropic action of PMS seems to involve a decrease in both follicular growth and luteinization. The histology of the ovaries following such inhibition does not seem to differ

TABLE 2. EFFECT OF A CONSTANT DOSE OF LAPACHOL ON THE RESPONSE OF THE OVARY OF IMMATURE RATS TO VARYING CONCENTRATIONS OF PMS*

Treatment			Ovarian wt. (mg.)†	% inhibition
PMS conc. (I.U.)	Lapachol (mg./day/4 days)	No. rats		
5	None	10	34.0±2.5	
5	6.0	18	23.4±2.2	31
10	None	10	76.8±3.8	
10	6.0	18	60.2±4.9	22
20	None	10	89.7±7.8	
20	6.0	20	68.0±4.3	24
40	None	60	152.6±5.2	
40	6.0	10	112.1±4.5	26
100	None	10	153.6±7.4	
100	6.0	10	93.6±8.7	39

* On the second day of quinone treatment each animal received the designated amount of PMS.
† Mean ±S.E.

from that of ovaries of equal weight produced by smaller doses of PMS.

The ability of certain 2-hydroxy-3-alkylnapthoquinones to act as potent inhibitors of respiratory processes has been reported by Ball *et al.* (6), and it seemed possible that this might be a contributory effect in the inhibitory action of lapachol on the response of the ovary to gonadotropins. It was found that lapachol alone had no effect on the succinoxidase activity of the ovary and that normal ovaries had a greater respiratory rate than those in which growth had been induced with PU or PMS (Table 3). The decrease in succinoxidase activity following response to gonadotropins has been explained by Meyer, *et al.* (12), as due to the diluting effect of the follicular fluid in the stimulated ovaries. However, there was a further reduction in the succinoxidase activity in ovaries from both PU and PMS treated rats injected with lapachol.

DISCUSSION

It is not possible at the present time to ascertain the exact manner in which quinones of the type proved active limit the ovarian response to PMS. There appear to be several pathways by which an agent such as lapachol might reduce the response of the ovary to PMS. One is chemical

TABLE 3. EFFECT OF GONADOTROPINS AND LAPACHOL ON THE SUCCINOXIDASE ACTIVITY OF RAT OVARIES

Treatment		No. determinations	$Q_{O_2}N$* (Mean ±S.E.)	P
Gonadotropin	Lapachol (mg./day/4 days)			
None	None	10	302±12	
None	6.0	10	291±10	>0.1
50 I.U. PU/day/3 days	None	10	254±12	
	6.0	10	227± 8	0.01
40 I.U. PMS	None	10	199± 7	
	6.0	10	161± 5	<0.02

* $Q_{O_2}N$—represents μl O_2 uptake/mg. nitrogen/hr. based on best 10 min. period after manometer taps were closed.

inactivation of the gonadotropin. If this occurs, then a large dosage of the inhibitory agent together with a minimal effective dosage of PMS would be expected to result in complete inactivation of the gonadotropin. Moreover, if a great surplus of PMS were administered with only a moderate dosage of inhibitor, then the effects of the inhibitor would be negated and a maximal ovarian response should occur. A second, and more plausible, explanation is that the inhibitory agent interferes with the growth processes in the ovary. This could explain the facts that large doses of PMS do not elicit a maximal ovarian response in lapachol-treated animals and that when a minimal effective dosage of PMS is given lapachol does not entirely depress the growth of the ovary. Thus, the phenomena observed seem best explained by the hypothesis that lapachol acts by limiting a metabolic process in the ovary rather than by reacting directly with the hormone. Though the specificity of action of lapachol may be questioned because of its toxicity, examination of Figure 1 indicates that lapachol inhibits ovarian growth only in PMS-treated rats and is without effect in PU-treated animals. However, since the degrees of PMS and PU ovarian stimulation are very divergent, non-specific toxicity still may be a partial explanation for the observed anti-gonadotropic activity of lapachol.

The inhibitory effectiveness of lapachol was tested on the response of the ovary to chorionic gonadotropin (Fig. 1), crude pituitary extracts, and response of the adrenal to ACTH. Lapachol did not depress the action of these hormones. Noble and Graham (9) also demonstrated that quinones do not readily inactivate chorionic gonadotropin when incubated with the hormone and injected at dose levels effective against PMS.

The powerful anti-respiratory activity of the napthoquinones might account for the inability of the PMS stimulated ovary to grow maximally. However, Figure 1 shows lapachol has no inhibitory effect on the ovarian action of PU, yet the succinoxidase activity of both PU and PMS stimulated ovaries are equally depressed by lapachol. In rats not treated with either PU or PMS, lapachol has no effect on ovarian enzyme activity.

The results summarized in Table 1 indicate a correlation of molecular structure with anti-gonadotropic activity. While trimethylquinone will inhibit PMS activity, introduction of an alkyl group at the six position reduces both the toxic effects and its inhibitory action in PMS stimulated rats. Structural requirements in another group are seen in the finding that lapachol could inhibit PMS, but that modification of the alkyl group by saturation of the double bond (hydrolapachol) or addition of a hydroxyl group at a terminal methyl (lomatiol) completely destroyed anti-gonadotropic activity.

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THE ANTITHYROID ACTIVITY OF 5-IODO-2-THIOURACIL[1]

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ABSTRACT

In tests with mature rats, thiouracil and 5-iodothiouracil (ITU) proved equally effective in suppressing thyroid uptake of iodide, and in suppressing organic binding of iodide by thyroid tissue. Intact ITU, synthesized with I^{131}, entered the thyroid both *in vitro* and *in vivo*, but no concentration gradient between thyroid and blood serum was found. The ability of a single thyroid to deiodinate ITU was negligible, but deiodination was appreciable in the whole animal. A mixture of thiouracil-KI increased the thyrotrophic activity of pituitaries of rats, while ITU did not. However, both thiouracil and ITU produced a small augmentation of exogenous thyrotrophin action, and both increased the biologic decay rate of thyroidal iodine. Measurement of urinary products after injection of radioactive ITU permitted an estimate of the contribution of released thiouracil and iodide to the antithyroid effect of ITU. It was concluded that ITU is an active antithyroid compound similar to thiouracil, but that the antithyroid effect is considerably modified by *in vivo* release of iodide from a portion of the administered drug.

THE ability of 5-iodo-2-thiouracil (ITU) to decrease both thyroid iodine content and oxygen consumption in rats, in quantities which produce minimal thyroid hyperplasia, has been previously described (1). Since the rat (2), dog (3) and human (4) are able to deiodinate some amount of ITU to produce thiouracil and iodide, it has been suggested that the antithyroid activity of ITU depends upon this conversion, and that the intact molecule has little, if any, activity (5). Our own results, judging by the quantities of ITU required to produce an antimetabolic effect, do not support this suggestion. Thus, it is possible to adjust dosages of thiouracil, or of thiouracil-KI, so that over a period of one or two weeks thyroid weight and iodine content resemble the values produced by a larger amount of ITU; however, in our experience, such dosages of thiouracil (or thiouracil-KI) do not lower the metabolic rate of rats. In view of these conflicting opinions, the present work was undertaken to obtain more direct evidence concerning the status of ITU as an antithyroid compound.

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MATERIALS AND METHODS

Mature Sprague Dawley rats, weighing 200–250 gm., were used for all tests. Carrier-free NaI^{131} was used for tissue and animal tests, and ITU containing radioiodine was prepared by releasing free iodine from NaI^{131} with acid permanganate, distilling it into an alkaline solution of 2-benzylthiouracil (6), and then removing the benzyl group with aluminum tribromide (7). Radioactive ITU (I*TU) with a specific activity of 1.48 μC./mg. was obtained, and its purity established by paper chromatography and radioautography. Whatman #40 paper and two solvent systems were used for all chromatography. Solvents used were (a) n-butanol/glacial acetic/water (34.5/9.6/16), and (b) n-butanol/ethanol, (1:1), saturated with 2N NH_4OH. Radioautographs were prepared by exposing the paper up to two months against No-screen X-ray film. Thyroids for *in vitro* tests were cut in two and incubated in Krebs-Ringer phosphate buffer at 37.5° C. Tissues were prepared for chromatography by digestion with Armour's pancreatin (8). Quantitative measurements of radioactivity were obtained in a deep-well scintillation counter, all samples counted in triplicate, and corrected for decay. Strips from paper chromatograms were sealed in glass tubes for counting. Iodine was determined chemically by dissolving thyroid tissue in 1 ml. of saturated KOH, drying, ashing at 400° C, and proceeding as described by Kirk (9). The alkaline ashing was entirely adequate when the weight of tissue did not exceed 50 mg. Drugs for injection were ground to a fine suspension in sesame oil, in such concentration that a single injection of 50 μM amounted to 0.1–0.2 ml. of suspension.

EXPERIMENTAL

1. *Uptake of I^{131} by thyroids. Organic binding of I^{131}.* Groups of 3 rats were given a single intraperitoneal injection of 50 μM. (6.4 mg.) of thiouracil or of ITU (12.7 mg.). One hour later 3 μc of NaI^{131} were given similarly,

TABLE 1. UPTAKE OF I^{131} BY RAT THYROID AFTER INTRAPERITONEAL INJECTION OF 3 μc. NaI^{131} AND 50 μM. OF THIOURACIL (6.4 MG.) OR OF IODOTHIOURACIL (12.7 MG.)

Time after I^{131} hours	% of total I^{131} ± stand. dev.		
	Control	Thiouracil	Iodothiouracil
½	8.5±0.67	1.4±0.11	1.3±0.10
1	14.5±1.2	1.2±0.09	1.5±0.12
4	28.2±1.74	1.5±0.09	1.0±0.04
8	32.0±2.7	1.5±0.11	1.9±0.13
24	29.0±2.6	3.7±0.28	1.1±0.10

and thyroid uptake of I^{131} measured at intervals thereafter on excised glands dissolved in KOH. The results of these measurements are shown in Table 1. It seemed most probable that suppression by ITU at 1.5 and at 2 hours was due to the intact molecule, but that later measurements would be affected by isotope dilution because of release of iodide from ITU. The effect of thiouracil and of ITU on organic binding of iodide *in vitro* was determined by incubating 9 samples of 4 halved thyroids each in 1.5 ml. of buffer containing 1 μc. NaI^{131}, and, except for control samples, a 10^{-3} M concentration of drug. At intervals, samples of tissue were removed, homogenized and counted. The homogenates were then precipitated and centrifuged 3 times in cold 10% trichloracetic acid. This procedure was

TABLE 2. ORGANIC BINDING OF I^{131} BY RAT THYROID SLICES IN BUFFER WITH DRUG CONCENTRATION 10^{-3} M.

Incubation time, hours	% of total I^{131}/100 mg. slices ± stand. dev.		
	Control	Thiouracil	Iodothiouracil
½	15.9±1.3	1.6±0.1	1.7±0.2
1	42.9±3.8	3.1±0.2	1.6±0.1
3	46.4±3.9	3.1±0.2	3.9±0.3

found to remove over 99% of inorganic iodide added to homogenized thyroid. The tissue residue was dissolved in KOH and counted to determine organically bound I^{131}. The results are shown in Table 2. Radioactivity remaining in tissue supernatant and buffer consisted of inorganic I^{131}, and essentially all the radioactivity was accounted for. No significant difference was found between thiouracil and ITU in either experiment.

2. *Uptake and deiodination of I*TU by thyroid tissue. Metabolic products of I*TU.* Presumably, in order to produce an antithyroid effect, it is necessary for a thiopyrimidine to enter the cells of the thyroid gland. Hence, in the previous *in vitro* experiment, ITU must have entered the gland, but the same results would have been obtained if the thyroid had rapidly converted ITU to thiouracil and iodide. To test both the uptake and deiodination of ITU by the thyroid, 3 samples, each composed of 4 halved thyroids, were incubated in 1.5 ml. of buffer containing 10^{-3} M I*TU. At ½, 1 and 3 hours tissue samples were removed, washed in 0.1N HCl, digested in pancreatin and counted. Two-month radioautographs prepared from paper chromatograms of the digested tissue showed no radioactive components except intact I*TU. Hence, tissue counts were calculated as I*TU, and the results are shown in Table 3. Radioactivity remaining in tissue washings and buffer consisted also of unchanged I*TU, hence suppression of organic binding of iodide by ITU in the previous experiment must have been due to the intact drug. The amount of drug taken up by a single thyroid was sufficient to contribute measurably to total thyroid iodine as determined chemically, and represented about a 2-fold concentration over that of buffer medium. Although deiodination of I*TU was not found in this experi-

TABLE 3. UPTAKE OF I^{131} IODOTHIOURACIL (I*TU) BY RAT THYROID AFTER INCUBATION *in vitro* WITH 768 μg. OF DRUG, OR AFTER INTRAPERITONEAL INJECTION OF 50 μM. (12.7 MG.) INTO INTACT RATS

Hours	*In vitro*			*In vivo*		
	Mg. of thyroid. (4 rats)	I*TU μg./100mg. thyroid	I*TU μg./gland	Mg. of thyroid (4 rats)	I*TU μg./100 mg. thyroid	I*TU μg./gland
½	52	18.2	2.4	—	—	—
1	57	38.6	5.5	69	9.4	1.6
3	47	49.5	5.9	62	29.4	4.6
8	—	—	—	57	22.8	3.3
24	—	—	—	68	14.9	2.9

ment, when the weight of tissue was increased to 100 mg., this amount of thyroid, liver, kidney or pituitary, live or boiled, produced a slight deiodination in 3 hours with the pituitary about twice as active as other tissues tested. Since iodide was liberated from the drug by incubation with cysteine at pH 7.4, deiodination in the tissues may depend only on the chemical action of free sulfhydryl groups.

For *in vivo* measurements, 16 rats were each given a single intraperitoneal injection of 50 μM. (12.7 mg.) of I*TU, housed in metabolism cages equipped with plastic funnels, and urine collected under toluene. At 1, 3, 8 and 24 hours after the injection, the thyroids of 4 animals were removed, and weighed and radioactive constituents determined by chromatography, radioautography and scintillation counting. The amount of I*TU found in the thyroids at different times is shown in Table 3 also. The smaller accumulation of I*TU at 3 hours in the thyroids *in vivo* was most probably due to binding of I*TU by the proteins of blood and other tissues. Of the total radioactivity 3 hours after injection, 80% was intact I*TU, and 17% (about 0.45 μg./gland) was I^{131} iodide. At 8 and at 24 hours, a small amount of radioactive thyroxine was also present. Radioactive constituents present in 24-hour urine are shown in Figure 1, which shows also the resolution of these substances in the acidic chromatographing solvent. Three of the constituents found in 24-hour urine (5-iodo-2-methylthiouracil, iodothiouracil and iodouracil) were found in 3-hour serum, in the relative proportions 11.4:75.1:13.3. Radioactive iodide was not present in detectable amounts. Iodopyrimidines were identified by eluting the radioactive areas from the paper and rechromatographing separately with known carriers. Identification was accepted when ultraviolet absorption and radioactivity coincided in both the acidic and basic solvents. The unknown component at R_f 0.33 is probably not a pyrimidine. The polarity indicated by its position on the paper suggests a salt or salt-like complex of iodine

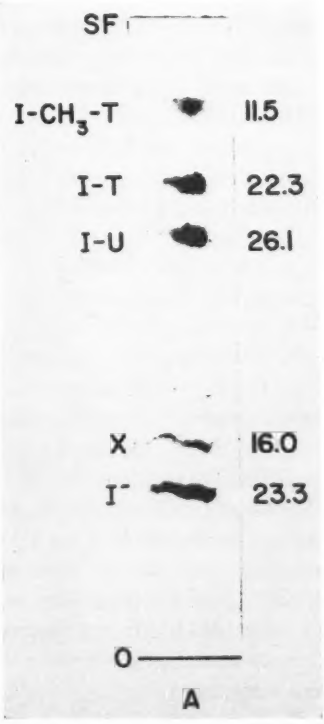

FIG. 1. Radioautograph from chromatogram of 24-hour sample of rat urine after administration of I*TU. I—CH_3–T: 5-Iodo-2-methylthiouracil. I–T: Iodothiouracil. I–U: Iodouracil. X: Unknown component. I^-: Iodide. Numbers at right give per cent contributed by each component to total radioactivity in urine.

formed after removal from a pyrimidine. Presence of the 2-methyl derivative was unexpected, but not surprising in view of the importance of methylation in detoxication processes. Of the total radioactivity injected as I*TU, 55% was excreted in 8 hours and 77% (63–90%) in 24 hours. If it is assumed that the unknown component was formed from released iodide, then the total iodide released from I*TU in 24 hours amounted to very nearly 2 mg., plus a few μg., either free or combined, remaining in the tissues. Since the equivalent weights of thiouracil and iodine are almost the same, the amount of thiouracil released must have been somewhat less than 2 mg., since the other iodopyrimidines formed from ITU are also deiodinated in the rat (2) and would have contributed to the total iodide. When I*TU was injected after dissolving in phosphate buffer, so as to increase the rate of absorption, excretion of radioactivity was more rapid. However, the per cent of I*TU excreted was larger, and that of I^{131}-iodide was smaller than when oil suspensions were injected.

At 3 hours, when accumulation of I*TU in the thyroids of intact animals was at or near maximum, 100 mg. of whole thyroid contained 29.4 μg. of drug, and 100 mg. of whole serum contained 32.3 μg. At 24 hours, the respective values were 14.9 and 13 μg. Hence, in the presence of a constant amount of drug these two tissues would show no concentration gradient. The small differences found at 3 and at 24 hours would be necessary for diffusion of drug between the two tissues during its absorption and excretion. Since excretion of the drug was not complete at 24 hours, some cumulation in the thyroid and other tissues is possible during chronic treatment. Pituitaries at 24 hours showed 3 times as much radioactivity as serum, but this amount was too small for identification of constituents. Radioactivity in adrenal, liver, spleen, leg bone marrow and submaxillary gland was less at both 3 and 24 hours than in the serum.

3. *Effect of ITU and of thiouracil-KI on thyrotrophic activity of rat pituitary.* The method used was adapted from that of Adams and Purves (10). Groups of 16 rats were injected subcutaneously each day with 50 μM. of ITU or with 50 μM. each of thiouracil and KI. After 14 days, pituitaries were removed, homogenized in saline, centrifuged, and the supernatants used for injection. Groups of 4 guinea pigs were maintained for 5 days on subcutaneous injections of 15 μg. L-thyroxine daily to suppress thyrotrophin (TSH) secretion, and on the second day given 20 μc. NaI^{131} intraperitoneally to label the thyroid hormones. On the 5th day, each guinea pig was given 2 intraperitoneal injections, 12 hours apart, each representing the supernatant from 1.5 rat pituitaries. Twelve hours after the second injection the animals were sacrificed, the thyroids and trachea removed, dissolved in base and counted. Results of this assay are shown in Table 4. Calculations are based on counts per minute (CPM) per thyroid gland. In view of qualitative similarities found between thiouracil and ITU in other measurements, it was expected they would show similar rather than oppo-

site effects on pituitary TSH. However, because of quantitative differences in potencies, the effects of the two drugs may have resulted from a combined time and dosage effect on pituitary TSH. Thus, D'Angelo has reported that 0.1% propylthiouracil in the diet of guinea pigs for 7 days caused a 75% decrease in pituitary TSH, while the same diet for 40 to 80 days caused increases of 280–1000% (11). This drug has also been reported to increase the TSH activity of rat pituitary (12). To test the effect of ITU and of thiouracil on exogenous TSH, 50 μM. of one of these drugs was injected into 4 guinea pigs (previously treated with NaI^{131} as previously described) along with 1.5 guinea pig units of Armour's Thytropar. The TSH injection was repeated after 8 hours, the animals sacrificed after 24

TABLE 4. EFFECT OF RAT PITUITARY EXTRACT ON THYROIDAL I^{131} OF GUINEA PIGS

Pituitary source	CPM/gland ±stand. dev.	% change
1. None	2430 ± 271	—
2. Normal rats	1171 ± 152	Group 1 vs. 2: −51.8 P<0.001
3. ITU-treated rats	1645 ± 198	Group 2 vs. 3: 40 P<0.01
4. Thiouracil + KI treated rats	515 ± 77	Group 2 vs. 4: −56 P<0.001

hours and the thyroids examined for radioactivity. Compared with control animals, those injected with TSH alone showed a 41% increase in loss of thyroidal iodine, while those injected with TSH plus either drug showed a 55–58% increase. The amount of augmentation was small, but fairly consistent for either drug. The effect of these drugs on the biologic decay rate of thyroidal iodine over a 24-hour period was determined as described by Albert and Ford (13). As reported by these workers, both drugs caused similar and significant increases in the biologic decay rate, but only those results obtained during the first few hours could be ascribed to intact ITU for animals treated with this drug. However, the released iodide may not have been important in altering the decay rate, since Wolff has reported that pharmacological dosages of KI fail to affect the release of I^{131} from thyroids of rats treated with TSH (14).

DISCUSSION

The ability of intact ITU to suppress thyroid uptake and organic binding of iodide (hence, hormone formation) renders inarguable the status of this compound as an antithyroid substance. When added to thyroid tissue *in vitro*, the resulting suppression can be ascribed only to the intact molecule, since the deiodination of ITU by a single rat thyroid is negligible. When administered to the whole animal however, it appears that the method of administration is of primary importance in determining the respective contributions of ITU and of the released thiouracil to the resulting antithyroid effect. Thus, as shown by the urinary products, when an effective dosage of ITU is administered to a 200–250 gm. rat in a single

injection, the amount of thiouracil released during the ensuing 24 hours is inadequate to produce a significant antithyroid effect in animals of this size. When the drug is injected as an aqueous solution, rather than as an oily emulsion, the more rapid absorption and excretion results in a still smaller release of iodide and thiouracil. A previous study of dehalogenation (2) has shown that increasing the dosage of ITU does not result in a corresponding increase in deiodination. The ability of the rat to deiodinate this compound is distinctly limited, hence it is probable that the rate of deiodination found in this work is close to the maximum rate for rats. Therefore, any antithyroid effect produced in this species by single daily injections of ITU (including the increased goitrogenicity of larger dosages) must be ascribed largely to the intact substance. Conversely, if the same amount of ITU is given in small divided dosages, at the same rate of deiodination a larger total quantity of ITU would be converted to thiouracil, and the latter would make a larger contribution to the antithyroid effect. Under these conditions, the behavior of ITU would tend to resemble that of thiouracil-iodide mixtures given in the same manner. The results would not necessarily be identical, since small frequent dosages of iodide would be required to simulate the steady release of iodide from ITU in various tissues. Thus, Goldberg and Wolff (5) found that 1% ITU administered to rats via the diet produced the same effects on the thyroid and on pituitary histology as a 0.1% mixture of propylthiouracil and iodide. However, the amount of ITU given was much greater than is required to produce an antimetabolic effect (1).

So far as the thyroid itself is concerned, the most striking difference between ITU and equivalent mixtures of thiouracil-iodide are the lesser depression of total thyroid iodine content caused by ITU, and the lesser goitrogenicity of this substance. The first difference seems adequately explained by the presence of intact ITU in the gland after treatment with this drug. Thus, from an experiment in which equivalent antithyroid dosages were injected for two weeks, thyroid iodine values for controls, thiouracil-iodide and ITU-treated rats were respectively 8.54, 2.8 and 4.7 μg. /gland for 10 animals. Assuming on the basis of the present work that thyroids of ITU-treated animals contained an average of 3 μg. of drug, equivalent to 1.5 μg. of iodine, then thyroids of these animals contained only 3.2 μg. of available iodine. Hence, by this measurement, the antithyroid action of ITU is not especially weak. Contrary to the results of Goldberg and Wolff (5), we have never found the thyroids of rats treated with adequate amounts of ITU to contain more total iodine than those of comparable control animals, despite presence of ITU in the thyroids. Since iodide is known to ameliorate the goitrogenic effects of thiouracil derivatives generally, the weaker goitrogenicity of ITU is most reasonably explained as due to release of iodide *in vivo*, which probably occurs in most tissues. In the excretion experiment, the amount of iodide released was equivalent to

somewhat more than 3 mg. of potassium iodide per day. This amount is in the pharmacological range, hence sufficient to repress the activity of both the thyroid and pituitary (15).

A recent paper by Maloof and Soodak (16) describes some experiments with S^{35} thiouracil (S*TU) similar to the ones described here with I*TU. These workers found that surviving sheep thyroid slices did not metabolize S*TU, and no concentration gradient between thyroid and serum was found after intraperitoneal injection in rats. In these respects, the behavior of S*TU resembles that of I*TU. However, they found also no concentration gradient between sheep thyroid slices and Krebs-Ringer buffer for S*TU, and drug in the thyroids was readily released by reincubating the tissue in fresh buffer. In the present work, a concentration gradient for I*TU in favor of the thyroid was found under the same conditions, and only a small amount of the compound was released when the tissue was transferred to fresh buffer. These differences between the two drugs we ascribe to protein binding. In connection with other work, we have found that either ITU or iodouracil is much more extensively bound by tissue and blood proteins at any pH above 3–4 than is thiouracil. Maloof and Soodak also reported a maximum thyroid uptake of S*TU within 30 to 60 minutes after injection in rats, while we found a maximum uptake of I*TU about 3 hours after injection. However, in addition to the difference in protein binding by these compounds, the S*TU was injected as an aqueous solution rather than as an oily suspension, so that absorption and distribution must have been more rapid than was found for I*TU.

It is concluded from the present work that intact ITU is an active antithyroid compound, similar to thiouracil in its activity and mechanism, but that the antithyroid effect is strongly modified in certain respects by the iodide released from a portion of the injected drug, and by the manner of the release. Extensive protein binding may also affect the activity of ITU. It may be noted that denying antithyroid activity to intact ITU because of the 5-polar substituent is no longer valid in the light of recent findings (17). Moreover, when attached to this position in the thiouracil ring, iodine is at best a weakly polar substituent.

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STATISTICAL RELATIONSHIPS AMONG METABOLIC PARAMETERS IN STEROID DIABETIC GUINEA PIGS[1]

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ABSTRACT

In normal guinea pigs, body weight was related to food intake, but neither was related to urinary non-protein nitrogen excretion. After the induction of cortisone diabetes, glucose utilization did not change significantly, the glycosuria being fully compensated. At this time, multiple regression analysis showed glycosuria to be significantly related to intake and nitrogen excretion, but not to weight; nitrogen excretion was related to glycosuria and weight, but not to intake.

HAUSBERGER and co-workers (1–3) have described certain metabolic changes produced in guinea pigs by the chronic administration of large amounts of cortisone and cortisol. However, they did not report on the statistical nature of the relationships among the several variables recorded. The present paper details such an investigation, using multiple regression techniques (4), as we reported previously for alloxan diabetes in rats (5).

MATERIALS AND METHODS

Nineteen adult guinea pigs were placed in individual metabolism cages and offered Rockland Guinea Pig Diet and tap water ad libitum. On analysis, this food was found to contain 3.2% fat, 22.6% protein, and 31.5% carbohydrate. Greens were offered twice weekly. After a 12-day period of acclimatization, during which time body weights were observed daily, measurements were begun of the daily food intake, urine volume, and urinary concentrations of glucose and non-protein nitrogen. Urine was collected in graduates containing thymol and sufficient citric acid to maintain the final pH below 3. After 7 days, the animals were given 2 subcutaneous injections of cortisone acetate each weekday and single injections on week ends, for average daily doses of 7.5 mg./day for 15 days, followed by 15 mg./day for 7 days. Four animals failed to develop a significant glycosuria and were eliminated from the experiment. We had previously been unsuccessful in an attempt to produce surviving diabetic guinea pigs by starting the injections at 20 mg./day. This indicated the necessity of following Hausberger's procedure (1–3) of increasing the cortisone dosage gradually. Over the course of the entire experiment, the room temperature ranged from 21–25° C and the humidity from 18–48%.

RESULTS

For statistical analyses, we utilized the raw data from the 15 diabetic animals during the 7 pre-cortisone control days and during the last 7 days

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TABLE 1. METABOLIC MEANS ±S.E. BEFORE AND AFTER CORTISONE

Metameter, per diem	Before	After	Change
Body weight, kg. (W)	0.877 ±0.0246	0.837 ±0.0277	−0.0405 ±0.0106**
Carbohydrate intake, gm. (I)	14.2 ±0.84	16.7 ±1.24	2.44 ±1.56
Glycosuria, gm. (G)	0.0 ±0.0	5.01 ±0.561	5.01 ±0.561**
Urinary NPN, ×3.65 gm. (N)	2.51 ±0.146	3.34 ±0.202	0.835 ±0.295*
Glucose utilization, gm. (U)	16.76 ±0.870	15.01 ±0.963	−1.75 ±1.34

* $P<0.05$
** $P<0.01$ that change = 0.

of cortisone treatment, disregarding the 15 days of increasing dosage and development of diabetes. The data were averaged over these two 7-day periods for each guinea pig. Food intake (I) was expressed as a gm. available glucose (i.e., 31.5% of the food eaten), glycosuria (G) as gm., urinary non-protein nitrogen (N) as gm. ×3.65, and body weight (W) as kg., all in terms of per guinea pig per day. Glucose utilization (U) was defined as $U=I+N-G$, with simplifying assumptions the same as those of Hausberger (1–3). The over-all means and associated standard errors are given in Table 1 and the matrices of the sums of squares and cross-products in Table 2. Statistical procedures and symbols have been adopted from Snedecor (4).

Control Period. Intake (I) and body weight (W), in the units described above, were significantly related ($r=0.63$*); the regression equation was $\hat{I}=14.24+21.466$ (W—0.87713). When the correlation coefficients of N and I or N and W were calculated, N was found to be independent of both I and W ($r_{NI}=0.11$, $r_{NW}=0.48$). These results suggested that body weight determined intake (or vice versa), but that urinary nitrogen excretion was not determined by either. The calculated glucose utilization was 16.8 ±0.87 gm./guinea pig/day, which corresponded to approximately 70 Calories from carbohydrate and protein.

Cortisone Period. The correlation coefficients were: $r_{IW}=0.74$, $r_{NI}=0.72$, $r_{NW}=0.72$, $r_{GI}=0.82$, $r_{GW}=0.67$, $r_{GN}=0.80$. Because they were all significant ($P<0.01$), the interdependence of the variables was removed by calculating partial coefficients, first taking G as the dependent variable, and then N. The partial regression coefficient of G on W (i.e., independent of any influence of N and I) was not significant and therefore W was deleted, reducing the equation to: $G=-2.90+0.235I+1.19N$. The partial regres-

TABLE 2. MATRICES OF SUMS OF SQUARES AND CROSS-PRODUCTS

	Control				Cortisone			
	W	I	N		W	I	N	G
W	0.127660	2.7403	0.35942	W	0.161444	5.3483	0.84111	2.20403
I		148.80	2.829	I		320.28	37.673	120.190
N			4.4786	N			8.5518	19.0548
				G				66.9389

TABLE 3. ANALYSIS OF VARIANCE OF REGRESSION OF G ON I AND N

Source of variation	d.f.	SS	MS	F
Total	14	66.939	4.781	
Regression				
On I and W	2	50.973	25.486	
On I alone	1	45.103	45.103	
On N alone	1	42.457	42.457	
Error	12	15.966	1.330	19.2**

sion coefficient of N on I (i.e., independent of any influence of G and W) was also nonsignificant, reducing this equation to: $N = 0.303 + 0.206G + 2.404W$. In other words, glycosuria was only indirectly related to body weight, and nitrogen excretion was only indirectly related to food intake, within the limits of our experiment. With regard to the cortisone period, we may postulate for the sake of clarity, without arguing causality or considering other unmeasured factors, that weight "determined" both intake and nitrogen excretion, and that intake "determined" glycosuria. It seemed reasonable that the *pari passu* changes in glucose and nitrogen excretion could be referred to some common factor resulting from the internal hormonal imbalance. The calculated glucose utilization in this period was 15.0 ± 0.96 gm./day, which corresponded to some 60 Calories from carbohydrate and protein.

DISCUSSION

The metabolic means in Table 1 show that cortisone caused a significant glycosuria, loss of body weight, and increase in nitrogen excretion. There was a slight, but non-significant, increase in intake and decrease in glucose utilization. Thus, we were able to agree with the statements of Hausberger and co-workers (1–3) that cortisone produced an increase in food intake and nitrogen excretion without changing the calculated glucose utilization, and that food intake was related to glucose and nitrogen excretion, but not to steroid dosage. (We have not documented the last point in the present report.) However, we could not confirm Hausberger's statements that there was an increase in body weight and that nitrogen excretion was unrelated to glycosuria or body weight, nor did we find any statistical basis for the use of a "nitrogen/food ratio."

The important point endocrinologically, as emphasized by Hausberger,

TABLE 4. ANALYSIS OF VARIANCE OF REGRESSION OF N ON G AND W

Source of variation	d.f.	SS	MS	F
Total	14	8.552	0.611	
Regression				
On G and W	2	5.935	2.967	
On G alone	1	5.424	5.424	
On W alone	1	4.382	4.382	
Error	12	2.617	0.218	13.6**

is that cortisone diabetes in guinea pigs is fully compensated, there being no significant alteration in the calculated glucose utilization. In our experience, this was true in spite of a declining body weight. These conclusions stand in contrast to the findings of Ingle (6), Franckson and co-workers (7), and others that glucose utilization is decreased in steroid diabetic rats. It is also dissimilar to the situation in alloxan diabetic rats (8).

The mathematically-inclined reader will recognize that the definition of U was arbitrary and that, by the method of discriminant functions (9), a set of coefficients for I, N, and G other than 1.0 can be derived that will better discriminate between the pre-cortisone and cortisone values of U. The matrices of sums of squares and cross-products given in Table 2 are necessary for this operation.

It should be emphasized that the method of multiple regression analysis we have employed is not entirely legitimate. This method assumes that such factors as weight, intake, nitrogen excretion, and glycosuria are independent variables, which they are not. However, multiple regression does allow for the uncovering of certain qualitative relationships that remain hidden in the raw data and that could be quantified legitimately only by such involved procedures as multivariate analysis.

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CORTISONE RESISTANCE IN PREGNANT RATS[1]

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ABSTRACT

It has been demonstrated, using doses of cortisone of 5 to 20 mg. per day, that pregnant rats exhibit a marked resistance to the biochemical effects of cortisone administration. This resistance was evident with respect to changes in alanine-glutamic transaminase activity, blood amino nitrogen and sugar levels and nitrogen balance data. There was no apparent resistance to the increase in liver weight produced by the hormone. Removal of the fetus alone did not affect this resistance whereas removal of both the fetus and placenta abolished it. It has been concluded that the placenta is essential for the maintenance of the resistance to the effects of cortisone seen in the pregnant rat. The possible mechanisms of this resistance are considered.

PREGNANT rats exhibit an increased anabolism of protein as indicated by biochemical alterations and net gains in tissue protein (1). Because of the well known protein-catabolic effects of cortisone, administration of this hormone to pregnant rats might be expected to reverse or at least impair this anabolism with a consequent adverse effect on gestation. By this procedure, abortion and macerated fetuses can be produced in rabbits and mice (2–4); doses of cortisone insufficient to produce abortion may produce congenital abnormalities (5, 6). However, in the rat, doses of cortisone as high as 25 mg./day produced no significant effect on the course of gestation (7). These studies have noted the gross effects of cortisone administration during gestation, but the biochemical effects have not been described. The present experiments deal with an investigation of the biochemical effects of the hormone and the nature of the resistance to these effects as seen in the pregnant rat.

METHODS

Nonpregnant and pregnant Wistar strain rats from the Carworth Farms colony having initial body weights between 190 and 220 gm. were housed in individual cages in a room maintained at 75±2° F and were provided with fresh water *ad libitum*. A 20% casein, 20% corn oil diet (8) supplemented with 60 mg. of α-tocopherol per kilogram was employed. In Experiments 1 and 4, the diet was fed *ad libitum;* in Experiments 2 and 3, the control animals were pair-fed with comparable pregnant or nonpregnant cortisone treated groups. Nitrogen balance studies were conducted from the 14th to the 17th days

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of gestation on small numbers of animals. Cortisone[2] was administered by single daily subcutaneous injections. Control animals received injections of 0.9% saline. No injections were given on the day of killing.

Animals were killed on the 21st day of gestation by stunning and decapitation following an 18 hour fast. The following assays were carried out on heparinized neck blood: urea (9), amino nitrogen (10, 11), sugar (12), packed cell volume. Liver aspartic-glutamic transaminase activity was measured by the method of Tonhazy *et al.* (13). The activity of the alanine-glutamic enzyme was measured by this method as modified by Caldwell and McHenry (14).

Fetuses were removed, counted and weighed at the end of each experiment. Fetal composition was determined in one experiment. The moisture content was determined by drying in open aluminum dishes for 5 hours at 105° C; protein was determined by the standard macro-Kjeldahl procedure; total crude fatty acids were determined by the method of Gavin and McHenry (15).

Statistical analyses were carried out by means of the "t" test. The significance of the difference between means is indicated by the value of "P".

RESULTS

The Effects of Cortisone Administration

In Experiment 1, the cortisone dosage was 5 mg./rat/day; all animals were fed *ad libitum*. This dosage was sufficient to cause a decreased food intake and a body weight loss. Therefore, when the dosage was increased to 7.5 mg./rat/day in Experiment 2 and to 7.5 mg./100 gm. body weight/day in Experiment 3, the pair feeding technique was employed to avoid this difference between cortisone and control groups. In these three experiments the injections were started on the 8th day of gestation and continued to the end of the study. The results from these experiments are shown in Table 1.

A dosage of 5 mg./rat/day produced a marked elevation of liver alanine-glutamic transaminase activity and of blood sugar levels in nonpregnant rats. However, neither of these alterations were seen in pregnant rats. Both pregnant and nonpregnant rats showed a decreased body weight gain and increased liver weight when treated with the hormone. Cortisone had no effect on the litter size or survival. The average placenta weight was reduced by the hormone (cortisone $= 0.33 \pm .01$ gm., saline $= 0.44 \pm .04$ gm., $P < .01$) but the fetus weight was unaffected.

When the dosage was increased to 7.5 mg./rat/day in Experiment 2, the food intakes of the pair-fed groups were as follows (gm./rat/day): pregnant $= 12.2$, nonpregnant $= 10.1$. Body weight gains were still reduced by the hormone in spite of pair feeding in both pregnant and nonpregnant rats; liver weight was again increased. Alanine-glutamic transaminase activity showed a marked response to the hormone in nonpregnant rats, but only a small response in pregnant animals. The aspartic-glutamic enzyme was not affected by cortisone nor was it affected by pregnancy. In the present experiment, no effect of the hormone on blood sugar was seen. A

[2] "Cortone Acetate" (courtesy of Merck and Company) lot KE-212 was used in the first experiment; lot KE-215 was used in all other experiments.

small but significant effect of the hormone on the average fetus weight was seen (cortisone = 4.07 ± .10 gm., saline = 4.93 ± .10 gm., $P < .01$); again there was no effect of the hormone on either litter size or survival.

In Experiment 3, the average food intake of the pair-fed groups was as follows (gm./rat/day): pregnant = 9.8, nonpregnant = 6.6. The hormone was administered in proportion to body weight in this experiment to counteract any effect of the difference in body weights of the pregnant and control rats at the end of gestation. The dosage used, 7.5 mg./100 gm. body

TABLE 1. THE EFFECTS OF CORTISONE ADMINISTRATION

(mean ± S.E.)

	Number of rats	Body wt. change (gm.)	Liver wt. (% body weight)	Liver transaminase[1]		Blood amino nitrogen (mg.%)	Blood sugar (mg.%)	Nitrogen balance[2] (mg./day)
				Alanine-glutamic	Aspartic-glutamic			
Experiment 1—5 mg./day								
Pregnant + cortisone	10	+25 ± 5	3.01 ± .08	38 ± 6	—	8.8 ± .3	37 ± 4	—
Pregnant + saline	7	+63 ± 6	2.68 ± .06	34 ± 6	—	8.8 ± .1	41 ± 3	—
Value of "P"		< .01	< .01	—	—	—	—	—
Nonpregnant + cortisone	12	−25 ± 3	3.79 ± .10	124 ± 6	—	9.6 ± .4	79 ± 3	—
Nonpregnant + saline	16	+11 ± 2	2.76 ± .11	58 ± 3	—	10.4 ± .2	57 ± 3	—
Value of "P"		< .01	< .01	< .01	—	—	< .01	—
Experiment 2—7.5 mg./day								
Pregnant + cortisone	11	+24 ± 5	3.10 ± .08	42 ± 5	120 ± 6	6.9 ± .2	36 ± 2	− 6 ± 22 (3)
Pregnant + saline	11	+69 ± 3	2.57 ± .05	26 ± 2	117 ± 5	7.5 ± .3	37 ± 3	+35 ± 14 (4)
Value of "P"		< .01	< .01	< .01	—	—	—	—
Nonpregnant + cortisone	9	−41 ± 2	3.83 ± .11	150 ± 7	122 ± 5	8.1 ± .2	77 ± 8	−50 ± 10 (5)
Nonpregnant + saline	9	+ 2 ± 3	2.63 ± .08	72 ± 4	116 ± 6	8.6 ± .2	70 ± 5	+17 ± 8 (4)
Value of "P"		< .01	< .01	< .01	—	—	—	< .01
Experiment 3—15–20 mg./day[3]								
Pregnant + cortisone	8	+ 4 ± 3	3.44 ± .07	70 ± 10	148 ± 15	9.5 ± .3	69 ± 5	− 1 ± 17 (3)
Pregnant + saline	6	+58 ± 9	2.62 ± .15	35 ± 5	146 ± 11	9.6 ± .6	63 ± 5	+25 ± 17 (4)
Value of "P"		< .01	< .01	< .02	—	—	—	—
Nonpregnant + cortisone	8	−49 ± 6	4.15 ± .12	141 ± 6	147 ± 14	9.4 ± .2	161 ± 19	−74 ± 23 (6)
Nonpregnant + saline	7	− 1 ± 6	2.66 ± .10	74 ± 14	130 ± 6	11.0 ± .3	87 ± 5	+24 ± 10 (5)
Value of "P"		< .01	< .01	< .01	—	< .01	< .01	< .01

[1] Transaminase activities are expressed as the μl. of pyruvate CO_2 formed per mg. wet tissue per hour.
[2] The numbers in parenthesis refer to the number of rats used in nitrogen balance studies.
[3] The actual dosage used was 7.5 mg./100 gm. body weight.

weight/day, provided an average dose of 20 mg./day for the pregnant rats and 16 mg./day for the nonpregnant rats. Again decreased body weight gains and increased liver weights were observed in both pregnant and nonpregnant rats. Alanine-glutamic transaminase activities were very high in nonpregnant rats. A significant elevation was produced in the pregnant rats also, but only to the normal nonpregnant level. Again it was seen that the activity of the aspartic-glutamic enzyme was unaffected by either cortisone or pregnancy. Blood sugar and amino nitrogen levels were significantly changed by the hormone in nonpregnant rats; no change in these metabolites was seen in the pregnant rats. With this very large dose of hormone, the fetus weight was markedly reduced (cortisone = 3.08 ± .29 gm., saline = 4.37 ± .24 gm., $P < .01$) although the litter size and survival remained unaffected. An analysis of the composition of the fetuses of these litters revealed that in spite of the weight reduction the compositions were unaltered. The actual compositions of the litters of cortisone and saline treated rats, respectively, expressed as per cent of total weight, were as follows: fat = 2.3, 2.0; protein = 9.3, 9.1; water = 87.1, 87.3. It is of interest to note that all of the pregnant rats treated with this large dose of cortisone

exhibited active lactation at autopsy. This was never seen in nonpregnant rats treated with this hormone, nor in pregnant rats treated with smaller doses of the hormone.

The results of nitrogen balance studies carried out with small groups of animals in Experiments 2 and 3 are shown in Table 1. With either dose of cortisone large losses of nitrogen were produced in nonpregnant rats; the magnitude of the negative nitrogen balance was increased with the larger amount of the hormone. In pregnant rats, the effect of the hormone on nitrogen retention was less severe. Even with the largest dose of cortisone, the nitrogen balance of the pregnant rats was only reduced to approximate equilibrium.

In all of these experiments, determinations of blood urea and of packed cell volume were carried out. Neither of these blood measurements was consistently altered by the hormone. Changes in blood constituents were not a result of changes in blood volume. Pregnant rats showed a normal hemodilution with no changes in blood urea.

The Role of Placenta and Fetus in the Resistance to Cortisone

In Experiment 4, the fetuses and placentas were removed from a group of rats by the method of Pritchard and Huggett (16) on the 16th day of pregnancy; the uterus was left *in situ*. This operation is referred to as complete cesarotomy. In a second group of rats, the fetuses only were removed, leaving the placentas *in situ*. In a third group of pregnant rats, the uterus was exposed and then replaced in the abdominal cavity. By similar treatment, a sham-operated nonpregnant group was obtained. After dividing each group, cortisone (15 mg./rat/day) or saline injections were started on the 17th day of gestation; all of the animals were fed *ad libitum*. Autopsy examination revealed complete survival of the placentas after removal of the fetuses although the size was not as great as in intact pregnant rats (fetus removed $= 0.26 \pm .02$ gm., sham-operated $= 0.36 \pm .01$ gm., $P < .01$). With only 4 days of hormone treatment in this experiment, cortisone did not decrease the average placenta weight, nor did it affect the average fetus weight.

Table 2 shows the results of determinations of alanine-glutamic transaminase activity, blood sugar levels and liver weights. No consistent effect was seen on blood urea, amino nitrogen or packed cell volume in this experiment. The actual values shown in Table 2 vary with the physiological conditions of the rats. However, when the differences produced by cortisone treatment are examined, a definite separation of the responses can be seen. All groups responded equally with respect to liver weight. With respect to transaminase activity and blood sugar, the responses of the nonpregnant and completely cesarotomized groups were of a greater magnitude than were the responses of the pregnant group and the group with the fetus removed but the placenta left *in situ*.

TABLE 2. THE ROLE OF THE PLACENTA AND FETUS IN THE RESISTANCE TO CORTISONE
(mean ± S.E.)

	Saline	Cortisone	Increase due to cortisone	Value of P
Liver alanine-glutamic[1] transaminase activity				
Pregnant	24 ± 2	43 ± 7	19	<0.05
Fetus removed	28 ± 3	44 ± 4	16	<0.01
Fetus + placenta removed	34 ± 6	100 ± 6	66	<0.01
Nonpregnant	51 ± 7	117 ± 13	66	<0.01
Blood Sugar (mg.%)				
Pregnant	35 ± 4	46 ± 4	11	—
Fetus removed	49 ± 5	60 ± 4	11	—
Fetus + placenta removed	55 ± 5	73 ± 5	18	<0.05
Nonpregnant	51 ± 5	75 ± 9	24	<0.05
Liver Weight (% body weight)				
Pregnant	2.44 ± .07	3.01 ± .08	0.57	<0.01
Fetus removed	3.33 ± .13	3.86 ± .09	0.53	<0.01
Fetus + placenta removed	2.77 ± .09	3.32 ± .10	0.55	<0.01
Nonpregnant	2.51 ± .14	3.05 ± .14	0.54	<0.02
Number of Rats				
Pregnant	6	6		
Fetus removed	6	7		
Fetus + placenta removed	6	7		
Nonpregnant	7	7		

[1] Transaminase activities are expressed as the μl. of pyruvate CO_2 formed per mg. wet tissue per hour.

DISCUSSION

Normally, the pregnant rat exhibits definite biochemical alterations after the 15th day of gestation (1). Among these alterations are a lowered liver alanine-glutamic transaminase activity, lowered blood amino nitrogen, increased liver weight and increased nitrogen retention. Although there is evidence of a lowered urea formation, blood urea remains unaltered (8). These findings were confirmed by observations on the saline treated rats in the present experiments. An additional finding was a consistent lowering of blood sugar during gestation. The role of anterior pituitary growth hormone in the control of these alterations has been discussed elsewhere (17).

The administration of cortisone to nonpregnant rats caused several alterations in intermediary metabolism. Among these changes was an increased alanine-glutamic transaminase activity. Beaton *et al.* (18) have associated an increase in the activity of this enzyme with an increase in protein catabolism. The extensive nitrogen loss produced by the hormone also gave evidence of an increased protein catabolism in the treated nonpregnant rats. However, on administration of the hormone to pregnant rats, a definite resistance to the biochemical effects of the hormone was seen. Decreased body weight gains and increased liver weights were still produced by the hormone, but even the highest dosage used failed to increase blood sugar levels or decrease blood amino nitrogen levels significantly. Very large doses of cortisone had to be employed to increase the alanine-glutamic transaminase activity to even normal non-pregnant levels. The protein

catabolic effects of the hormone were markedly reduced in the pregnant rat. The course of gestation was not interrupted by the hormone; fetus weights were reduced, but the composition remained unaltered and the fetuses were normal in appearance. Other workers have reported that the effect of the hormone on the fetus may result in death shortly after birth (19); this was not studied in the present experiment.

It has been reported (20) that the administration of cortisone will cause a reduction of the placenta weight; this effect was seen in the first experiment. A subsequent investigation of the role of the placenta and the fetus revealed that the placenta, but not the fetus, was essential for the resistance to the effects of cortisone. It has also been demonstrated (21) that the placenta, with or without the ovaries and fetuses left intact, is essential for the weight gain and nitrogen retention exhibited by pregnant rats.

The manner in which the placenta exerts these functions is not known. Many reports have appeared in the literature describing the presence of hormones in placental extracts. It has been postulated that the placenta is capable of synthesizing many hormones. If such is the case, the placenta may actually produce hormones to counteract the effects of cortisone. If this were true it would be expected that the placenta weight would increase during treatment rather than decrease. At least two other explanations exist: the placenta may influence the endocrine glands to produce additional hormones, or the placenta may have the ability to concentrate or destroy the administered cortisone. The present studies give no information about the method by which the placenta acts, but they do illustrate that the placenta is involved in the resistance to cortisone administration in the pregnant rat. It has been reported (22, 23) that pregnant women also exhibit a resistance to the effects of this hormone; the role of the placenta in the human resistance is not known. The results of other studies in this laboratory, to be published shortly, demonstrate that pregnant rats exhibit no resistance to the protein-catabolic effects of glucagon. The resistance to cortisone is not part of a general overall resistance to protein-catabolic compounds.

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SOME HORMONAL INFLUENCES ON THE ACETYLATION OF SULFANILAMIDE *IN VIVO*[1,2]

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ABSTRACT

The previously reported studies on sex differences in per cent acetylation of sulfanilamide by rats were extended *in vivo*. It was established that immature rats of both sexes acetylated to the same extent. Castration at, or near birth, and estradiol injections reduced the extent of acetylation by males. Adrenalectomy superimposed on castration had no greater effect than castration alone in either sex. Ovariectomy was without effect, whereas hypophysectomy lowered markedly the extent of acetylation by both males and females. The radioactive urinary acetylsulfanilamide isolated after injection of radioacetate and sulfanilamide was found to have a markedly greater specific activity in males than in females; the specific activity was also greater in normal males as compared to hypophysectomized males. The results are discussed in light of their possible reflections of two-carbon metabolism in the body.

THE enhancement of sulfanilamide acetylation *in vitro*, by pigeon liver, and *in vivo* in the rat by testosterone and testosterone propionate has been reported (2). Further clarification of this hormonal effect appeared justified in view of the participation of acetyl coenzyme-A in arylamine acetylation and the key position occupied by this two carbon fragment in metabolism. This communication extends the *in vivo* findings previously reported to include the effects of castration, adrenalectomy and hypophysectomy on this acetylation reaction in rats.

MATERIALS AND METHODS

All rats used were of the Sprague-Dawley strain. Animals used for early castration were bred in the laboratory; hypophysectomized animals and their controls were obtained from Hormone Assay Laboratories, Chicago, Illinois. All other rats were supplied by the Holtzman Rat Co., Madison, Wisconsin.

The animals were fed Rockland rat diet *ad libitum* or, in the case of the adult hypophysectomized rats and their controls, Hill's Horsemeat, supplemented with oranges, and Rockland diet. The adrenalectomized and castrated rats were maintained on 1.5%

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NaCl-5% glucose for one week postoperative, and on 1.5% NaCl thereafter. At the conclusion of the experiment tapwater was substituted for the NaCl solution. Only results from rats dying within the week after removal of the NaCl solution were included in the study.

All operations were performed with clean instruments, without aseptic conditions. Early castrations were performed within the first week *post partum* by the method of Pfeiffer (3) under low temperature anesthesia. Orchidectomies and ovariectomies either alone or coupled with adrenalectomy were performed on sexually immature rats, under ether anesthesia. Sham operations were performed under the same conditions.

Estradiol 17-β (Mann, Lot nos. 103519 and 1083282), and testosterone (U.S.P., Nutritional Biochemicals) were dispersed by sonic oscillation (2) or by homogenization in an all glass homogenizer in 0.9% NaCl and diluted to appropriate concentrations. The dispersed hormones were injected intraperitoneally (I.P.) daily for the stipulated time in one cc. portions. Control animals received injections of one cc. of 0.9% NaCl.

Sulfanilamide (Merck U.S.P. recrystallized three times from 50% ethanol) was injected I.P. into the lower quarter of the abdomen of the rat in one dose of 70 mg. per kilogram body weight (2). The urine was collected under toluene for twenty-four hours following the injection, diluted to one liter and appropriate aliquots were analyzed for free and total sulfanilamide according to the method of Bratton and Marshall (4), with minor procedural modifications, and using the Coleman, Jr. Spectrophotometer, Model 6A.

The extent of acetylation by the rat was recorded as "per cent acetylation," the ratio of the amount acetylated to the total amount recovered in the urine multiplied by 100 (2). Recoveries of total sulfanilamide ranged from 75–103% based on the injected dose. The "t" test was used to compare averages between control and experimental groups; a "t" value falling at the 5% level of probability was considered significant (5).

Sodium acetate-1-C^{14} (15 mg. per cc. per 200 gm. body weight, 44,444 cts./min./mg.; or 0.4 mg. per 0.5 cc. per 200 gm. body wt. 390,000 cts./min./mg.) was injected I.P. into rats and five minutes later 70 mg. per kilogram body wt. of sulfanilamide was injected, the urine collected for 24 hours following the administration of the sulfanilamide and diluted to 100 cc. One cc. was used for analysis as described above. The remainder of the urine was transferred quantitatively to a separatory funnel, a known amount of carrier acetylsulfanilamide (prepared from p-acetaminobenzene sulfonyl chloride (6) and recrystallized three times from 50% ethanol) in water solution was added. The urine was extracted with methylethylketone. The extract was evaporated to dryness and the impure crystals of acetylsulfanilamide recrystallized four to eight times from 50% ethanol in small test tubes. Melting points taken on each sample agreed within one degree of the literature values (217° C). The radioactive acetylsulfanilamide was counted in a Tracerlab Automatic windowless counter (Model SC50) with a Tracerlab scaler (Model 5C51R) and printer (SC5E) with a sufficient number of counts for a probable error of less than 5%. Original specific activities of the excreted, undiluted acetylsulfanilamide were calculated.

RESULTS

Immature males and females did not differ in the extent to which they acetylated sulfanilamide (Table 1). Early castration of male rats decreased significantly the per cent acetylation by these animals as compared to sham operated controls (Table 1).

Estradiol 17-β injected I.P. at a dose of 200 μg. per day for a period of 31–42 days significantly lowered the per cent acetylation of males (Table

TABLE 1. EFFECT OF IMMATURITY AND EARLY CASTRATION ON THE ACETYLATION OF SULFANILAMIDE *in vivo*

No. of rats	Conditions	Average weight† (gms.)	Determinations	Mean % acetylation	% change from controls	Statistical significance
13	Immature males*	68	13	54.6±6.8†	1.0	t=0.25
13	Immature females	94	13	55.2±4.9‡		P=0.8
13	Males castrated 0–6 days post-partum	156	23	60.0±6.7‡	8.9	t=3.06
5	Males shammed 0–6 days post-partum Tested 45–109 days post-operative	176	5	65.9±2.9‡		P=0.01

* Assumed as control group.
† Weight at time of determinations.
‡ Standard deviation from mean.

2). A short term experiment using smaller experimental and control groups suggested that this dose of Estradiol-17-β could lower the per cent acetylation within seven days past the beginning of the injection period. Removal of the ovaries of immature females did not appear to change the extent of acetylation 32 to 70 days postoperative. The control animals were sexually mature at the time of the test (Table 2).

Testosterone when injected I.P. into females at a dose of 500 μg. per rat per day for a period of 30–38 days had no significant effect on the level of acetylation when compared with the control group. When the dose of testosterone injected was increased to 1000 μg. per rat per day for an addi-

TABLE 2. EFFECT OF ESTRADIOL 17-β AND OF OVARIECTOMY ON THE ACETYLATION OF SULFANILAMIDE *in vivo*

No. of rats	Conditions	Average weight* (gm.)	Determinations	Mean % acetylation	% change from controls	Statistical significance
12	Males 200 μg. Estradiol 17-β I.P. 31–42 days	175	23	55.5±4.7†	16.7	t=9.35
12	Males 0.9% NaCl I.P. 31–42 days	267	24	66.7±3.5†		p=0.001
7	Males 200 μg. Estradiol 17-β I.P. 5–7 days	153	7	56.4±7.8†	13.8	t=2.39
6	Males 0.9% NaCl I.P. 5–7 days	180	6	65.6±5.9†		p=0.05
13	Females Ovariectomy 32–70 post op.	182	13	54.1±3.8†	4.2	t=1.73
11	Females Sham operation 32–70 post op.	195	11	51.9±2.7†		p=0.10

* Weight at time of determinations.
† Standard deviation from mean.

TABLE 3. EFFECT OF TESTOSTERONE ON THE ACETYLATION OF SULFANILAMIDE BY FEMALE RATS *in vivo*

No. of rats	Conditions	Average weight* (gm.)	Determinations	Mean % acetylation	% change from controls	Statistical significance
12	500 μg. Testosterone I.P. 30–38 days	181	12	52.8 ± 3.9†		t = 0.757
					2.5	
11	0.9% NaCl I.P. 30–38 days	183	11	54.2 ± 4.9†		p = 0.4
12	1000 μg. Testosterone I.P. 31–54 days	271	12	56.2 ± 2.6†		t = 6.22
					11.0	
11	0.9% NaCl I.P. 31–54 days	252	11	50.6 ± 1.7†		p = 0.001

* Weight at time of determinations.
† Standard deviation from mean.

tional 31–53 days using the same control and experimental animals a significant increase was observed (Table 3).

When adrenalectomy was imposed upon castration, thus removing another source of androgens, there was apparently little effect, if any, over and above that of castration alone in either sex (Table 4). However hypoph-

TABLE 4. EFFECT OF ADRENALECTOMY PLUS CASTRATION, AND OF HYPOPHYSECTOMY, ON THE ACETYLATION OF SULFANILAMIDE *in vivo*

No. of rats	Conditions	Average weight‡ (gm.)	Determinations	Mean % acetylation	% change from controls	Statistical significance
6	Males castration plus adrenalectomy, 56–72 days postoperative	326	6	51.2 ± 0.8§		t = 1.29
					3.0	
7	Males castration* only 56–72 days postoperative	242	7	52.8 ± 2.9§		p = 0.3
4	Females castration plus adrenalectomy, 95–99 days postoperative	339	4	50.1 ± 1.2§		t = 2.69
					1.2	
6	Females castration only 95–99 days postoperative	285	6	49.5 ± 1.6§		p = 0.6
12	Adult males hypophysectomized†	177	12	50.8 ± 6.8§		t = 6.59
					22.2	
12	Adult males	253	12	65.3 ± 3.3§		p = 0.001
12	Adult females hypophysectomized†	175	12	32.1 ± 5.0§		p = 0.001
					37.9	
11	Adult female controls	228	11	51.7 ± 5.0§		t = 10.3

* Assumed as control group.
† 14–26 days post op.
‡ Weight at time of determinations.
§ Standard deviation from mean.

ysectomy did lower the per cent acetylation significantly in both sexes (Table 4).

The incorporation of radioacetate into acetylsulfanilamide seemed to be far greater in males than in females and in hypophysectomized males. These data are preliminary in scope.

DISCUSSION

The data presented indicate that sexual maturity is necessary for the appearance of a male-female difference in the acetylation of sulfanilamide *in vivo* by the albino rat. The values presented for adult control groups of both sexes agree well with those presented in the original studies of Franz and Lata (2). Nearly two-thirds of the sulfanilamide recovered from normal males was acetylated in contrast to only half of that from females.

Although castration of fully mature males had no effect on their ability to acetylate sulfanilamide (2), the data in Table 1 show that early castration was effective in this respect. This would suggest that early castration successfully removes the main androgenic influence involved in the maintenance of a maximal degree of acetylation in males. Orchidectomy of the adult might remove the main *source* of androgenic steroids, but residual testosterone, or androgenic metabolites thereof, might remain in the circulation for some time afterwards. In sufficient dosage testosterone (Table 3) can raise the per cent acetylation of females. As previously reported (2) the extent of acetylation by females was increased by injection of 500 μg. of testosterone propionate in solution per day. In this study an equal dose of testosterone in suspension was ineffective (Table 3). The effect of double this dose was comparable to that of the aforementioned work of Franz and Lata. It is well known that the propionate is the more effective androgen (7).

A high dose (200 μg.) of estradiol 17-β depressed the per cent acetylation of males and was effective within a week following the first injection, whereas ovariectomy had no significant effect. The results in Table 4 strengthened the possibility of pituitary inhibition by high estradiol doses as a reason for estrogen inhibition of acetylation. These data agreed with the report (8) that hypophysectomy decreased acetylation of *p*-aminobenzoic acid by rats. Lowered liver coenzyme A levels in hypophysectomized rats (16) would also bear on these data.

This sex difference in the acetylation of sulfanilamide, and its control by sex hormones and an intact pituitary might be a reflection of the hormonal control of other acetyl coenzyme-A dependent reactions. Thus inhibitions by testosterone or stimulations by estrogens might be reflected in increased or decreased acetylations respectively. Observations presenting a consistent picture with the results herein when so interpreted have been reported: Deuel and his coworkers (9) have demonstrated the *in vivo* de-

pendence on ovarian secretions of induced ketonuria in the rat. Dubois and coworkers (10, 11) have shown that citrate accumulation by rat liver *in vivo* and *in vitro* is inhibited by testosterone. The data of Soodak and Lipman (12) indicated that acetoacetate formation competed with acetyl sulfanilamide formation when both were formed together *in vitro*. Acetylation by human red blood cells from patients in diabetic coma was virtually non-existent (13) and the acetylation of p-aminobenzoic acid by rats rendered diabetic was diminished (14). Ketosis is a well known part of the diabetic syndrome.

Whether or not the rates of the various enzymic reactions utilizing acetyl coenzyme-A were affected by the hormones in question or the effective concentrations of the apo-enzymes were changed by the known protein anabolic effects of androgens and anterior pituitary hormones (7, 15) cannot be decided by these data. Preliminary data (Table 5) on the incorporation of radioacetate into acetylsulfanilamide *in vivo* by normal male and female, and hypophysectomized male, rats suggested that the rate of turnover of acetyl coenzyme-A in the livers of male rats was greater than that of female rats or hypophysectomized male rats. The magnitude of the per cent change in incorporation is so much greater than the differences found in per cent acetylation that a hormonal effect on the arylamine acetylase enzyme is probably not the *sole* explanation. Similarly, were the pool size of acetyl coenzyme-A in the normal males larger than that in the females or controls then by dilution effects the differences in incorporation would have been qualitatively opposite to that of the results obtained. If the pool size in the females and hypophysectomized males were larger than that in normal males, it would be difficult to envision a greater extent of acetylation in the latter group unless the turnover rate of acetyl coenzyme-A were also substantially greater.

TABLE 5. INCORPORATION OF RADIOACETATE INTO ACETYLSULFANILAMIDE *in vivo*

No. of animals	Conditions and sex	Mean specific activity (cts. per min. per mg.)	% difference
3	Adult males	949*	
3	Adult females	330*	65
3	Control males	174†	
3	Hypophysectomized males	95†	45

* Initial dose 666,666 cts./min./200 gm. body wt.
† Initial dose 136,000 cts./min./200 gm. body wt.

These results could also be interpreted as a more direct effect on peptide bond formation, and thereby, protein synthesis, were the formation of acetylsulfanilamide considered a model for peptide bond formation. Acetylation of sulfanilamide, however, appears to be only weakly analogous to peptide bond formation.

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THYROXINE-LIKE COMPOUNDS AND CHOLESTEROL METABOLISM: DIFFERENCES IN THE EFFECTS OF THYROXINE, TRIIODOTHYRONINE AND THEIR FORMIC ACID ANALOGUES[1]

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ABSTRACT

The biologic activity of *l*-thyroxine, *l*-triiodothyronine, and their formic acid analogues, tetraiodothyroformic acid and triiodothyroformic acid have been studied in the rat. By thiouracil-goitre inhibition assay they were found to have the following relative activities (on a molar basis): *l*-thyroxine, 100%; *l*-triiodothyronine, 333%; tetraiodothyroformic acid, 0.08%; and triiodothyroformic acid 0.27%.

In addition to this quantitative difference, the physiologic activity of tetraiodothyroformic acid was also found to differ in other respects from that of *l*-thyroxine. When administered in amounts having equal goitre inhibiting activity, the formic acid analogue exerted significantly greater effect on cholesterol metabolism than did *l*-thyroxine. As compared to *l*-thyroxine, tetraiodothyroformic acid also exerted relatively greater effect on cholesterol metabolism than on growth.

MANY compounds structurally related to thyroxine have been observed to exert thyroxine-like activity. In comparing the degree of endocrine activity of these analogues to that of thyroxine itself various techniques have been employed. Measurements of acceleration of tadpole metamorphosis (1), oxygen consumption by the intact animal (2) or by tissue slices (3), and the inhibition of thyroid enlargement produced by goitrogens (4) are widely used methods of assay. These various techniques measure different physiologic functions altered by or dependent upon thyroxine. Interestingly the relative potency of thyroxine and some of its analogues is not the same by all assay methods (5).

These differences in relative potency, dependent upon the physiologic effect observed, suggest the possibility that one or more of the analogues of thyroxine might exert a disproportionate effect on cholesterol metabolism. A preliminary report indicated that the formic acid analogue of thyroxine, tetraiodothyroformic acid, exerted a relatively greater effect on cholesterol metabolism than on goitre resulting from thiouracil-feeding (6).

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The present communication reports further evidence for differences in the physiologic effects of thyroxine and its formic acid analogue. The effects of the administration of graded amounts of thyroxine and tetraiodothyroformic acid to the cholesterol-thiouracil fed rat are described. The relative activities of these compounds with regard to goitre-inhibition, growth promotion and cholesterol metabolism have been studied. Comparisons have also been made with triiodothyronine and its formic acid analogue, triiodothyroformic acid (Figure 1).

METHODS

Male albino rats (Holtzman) weighing approximately 300 gm. each were employed. They were housed in mesh bottomed cages and kept in an air-conditioned animal room at approximately 25° C. Food and tap water were provided *ad libitum*. Those animals receiving thyroxine or related compounds orally were kept in individual cages; those receiving them parenterally were caged in groups.

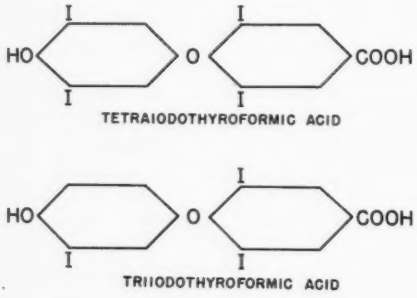

FIG. 1.

Two high cholesterol diets were employed. In the majority of the studies the diet consisted of Purina rabbit chow to which was added 1% cholesterol and 5% cottonseed oil. In some later studies a balanced laboratory mash (rat-mouse chow, Harlan Small Animal Industries, Cumberland, Indiana) was substituted for the rabbit chow. In the preparation of both diets the cholesterol was dissolved with the help of warming, and the sterol in oil sprinkled on the feed with constant stirring. When thiouracil was administered it was thoroughly mixed with the diet in the amount of 0.5% by weight.

The materials for administration were prepared as follows: Sodium *l*-thyroxine (Travenol) was dissolved in 1% aqueous sodium bicarbonate, the final pH being 8.8. *l*-Triiodothyronine (Smith, Kline, & French) was prepared in the same manner. Thyroformic acid, triiodothyroformic acid and tetraiodothyroformic acid[2] were prepared in a solution of 0.9% sodium bicarbonate and 0.1% sodium carbonate, the final pH ranging from 8.0 to 9.0. The thyroformic acid gave a clear solution, as did the triiodo analogue except at the highest concentration. The tetraiodothyroformic acid was incompletely dissolved, and was utilized as a fine suspension. Fresh solutions were prepared twice weekly and kept refrigerated. When administered parenterally the several solutions were injected subcutaneously each morning, the volume injected being approximately 0.25 ml. For oral administration the solutions were sprayed on the feed each day and air dried.

The animals were sacrificed following intraperitoneal injection of amobarbital sodium, and blood obtained by cardiotomy. The thyroid glands were carefully dissected free of fat and connective tissue, being protected against drying by moist filter paper, and weighed to the nearest 0.1 mg. in covered bottles.

The livers were removed, weighed, and placed in tightly covered jars containing 3 ml.

[2] The authors are grateful to Dr. Earle Van Heyningen, Lilly Research Laboratories, Indianapolis, Indiana, for the synthesis of the thyroformic, triiodothyroformic and tetraiodothyroformic acids.

per gram of liver of a 25% potassium hydroxide solution in 95% ethyl alchohol. The jars were placed overnight in a water bath at 37° C.

Serum cholesterol was determined by the method of Abell and associates (7). To 1 ml. of the liver digestant was added 5 ml. of 95% alcohol. Petroleum ether extraction and determination of cholesterol were than performed according to the method of Abell.

Hereafter the following designations will be employed: T-4 for *l*-thyroxine; T-3 for *l*-triiodothyronine; TFA-4 for tetraiodothyroformic acid; TFA-3 for triiodothyroformic acid; TFA for thyroformic acid.

RESULTS

Effects of T-4, T-3, TFA-4, TFA-3 and TFA on the cholesterol-thiouracil fed rat

Eighty-three animals, divided into groups of approximately equal mean weight, were studied for a 2-week period. All received one of the two high-cholesterol diets with 0.5%

TABLE 1. EFFECTS OF *l*-THYROXINE (T-4), *l*-TRIIODOTHYRONINE (T-3), TETRAIODOTHYROFORMIC ACID (TFA-4), AND TRIIODOTHYROFORMIC ACID (TFA-3) ON THE CHOLESTEROL-THIOURACIL-FED RAT[1]

No. rats	Compound and daily dose (μM/100 gm.)	Thyroid weight (mg./100 gm.)	Goiter inhibition[2] (%)	Serum cholesterol[3] (mg./100 ml.)	Liver cholesterol	
					Conc.[3] (mg./100 gm.)	Total[3] (mg.)
15	CONTROL	18.6 ± 3.3		153 ± 31	1126 ± 307	142 ± 38
7	T-4 0.0021	11.1 ± 4.0	60	114 ± 11**	856 ± 317	133 ± 51
7	T-4 0.0039	6.2 ± 1.4	100	122 ± 21*	744 ± 264*	108 ± 32
4	T-4 0.0078	3.6 ± 0.1	100+	128 ± 23	599 ± 260**	79 ± 29**
4	T-3 0.001	8.7 ± 4.4	80	110 ± 14*	663 ± 78**	89 ± 17**
8	TFA-4 1.36	14.9 ± 2.1	30	110 ± 20**	489 ± 148**	66 ± 27**
10	TFA-4 2.72	12.7 ± 2.9	48	106 ± 14**	375 ± 81**	48 ± 13**
4	TFA-4 4.08	8.8 ± 2.0	79	105 ± 14**	402 ± 157**	55 ± 17**
4	TFA-4 5.44	4.8 ± 0.1	100+	100 ± 13**	268 ± 10**	33 ± 3**
4	TFA-3 0.33	15.0 ± 2.5	29	127 ± 13	831 ± 271	123 ± 50
4	TFA-3 0.66	13.3 ± 3.1	43	146 ± 10	792 ± 111	135 ± 14
8	TFA-3 1.32	7.9 ± 2.1	86	108 ± 17**	615 ± 315**	98 ± 53*
4	TFA 2.72	19.2 ± 2.3	0	167 ± 24	1592 ± 416	159 ± 36

[1] Values given are the mean and standard deviation estimated using (n − 1).

[2] $\%\ \text{goitre inhibition} = \dfrac{\text{control thyroid wt.} - \text{observed thyroid wt.}}{\text{control thyroid wt.} - \text{normal thyroid wt.}} \times 100.$

[3] Values that differ significantly from the control (t test) are indicated as follows:

* = $.01 < P < .05$ ** = $P < .01$

added thiouracil. The control group of 15 animals received daily subcutaneous injection of 0.25 ml. 1% sodium bicarbonate solution. The remaining groups received daily subcutaneous injections as follows: T-3, 0.001; T-4, at three levels, 0.0021, 0.0039 and 0.0078; TFA, 2.72; TFA-3, 0.33, 0.66 and 1.32, and TFA-4, 1.36, 2.72, 4.08 and 5.44, all doses being expressed in micromoles per 100 gm. body weight. The animals were sacrificed at the end of the 2-week period and thyroid weight and serum and liver cholesterol determined.

The results are presented in Table 1. The control group consists of animals which received both types of high-cholesterol feed. Since mean serum and liver cholesterol concentrations and thyroid weights relative to body weight were essentially identical in the two control groups they are com-

bined in the Table. The administration of the experimental diet to the control group for 2 weeks, resulted in a three-fold increase in thyroid weight from the normal mean of 6.2 ± 0.9 mg./100 gm. observed in our laboratory. Serum and liver cholesterol levels are significantly increased as compared to the means of 80 ± 10 mg./100 ml. and 314 ± 30 mg./100 gm. liver observed in 14 noncholesterol-thiouracil fed rats ($P < .01$).

T-4, T-3 and their formic acid analogues all displayed goitre-inhibiting activity and, when administered in sufficient amount, exerted an effect on serum and liver cholesterol. Thyroformic acid was completely inactive in these respects. The approximate goitre-preventing doses (in μM per 100 gm. body weight) of the 4 compounds are as follows: T-4, 0.004; T-3, 0.0012; TFA-4, 5.0; TFA-3, 1.5.

TFA-4 differed from the other compounds in that at a similar degree of goitre inhibition it exerted a greater effect on liver cholesterol. The disproportionate effect of TFA-4 is equally apparent when liver cholesterol is expressed in absolute terms or as concentration.

Effects of T-4, TFA-4 and sodium iodide on the euthyroid cholesterol fed rat

Seventeen animals were divided into 4 groups of approximately equal mean weight. All received the high-cholesterol rabbit chow diet without added thiouracil. The controls received daily subcutaneous 1% sodium bicarbonate. The remaining 3 groups received subcutaneously: T-4, 0.0039; TFA-4, 2.72; and sodium iodide, 10.88 μM per 100 gm. body weight. Again the animals were sacrificed at the end of a 2-week experimental period, and thyroid weight and serum and liver cholesterol determined.

The effects of T-4 in full goitre-preventing dose and of TFA-4 in approximately one half of the goitre-preventing dose on the euthyroid cholesterol-fed rat are given in Table 2. The relatively greater effect of the formic acid analogue on liver cholesterol is also demonstrated by this study, both the concentration and total amount being significantly lower than in the animals receiving T-4 ($P < .01$). Sodium iodide in such dose as to provide the same amount of iodine as the TFA-4 employed was without demonstrable effect.

Effects of oral administration of T-4 and TFA-4 for 6–8 weeks

Twenty-seven rats, divided into 5 groups, were employed. All received the rabbit chow high-cholesterol diet with 0.5% added thiouracil. The control group received no further additive to the diet. To the daily food ration of each animal in the remaining 4 groups, one of the following additions was made daily: T-4, 0.0078 or 0.0156 μM; or TFA-4, 2.72 or 5.44 μM, all per 100 gm. body weight. The animals were weighed at weekly intervals. At the end of 6 weeks all animals were sacrificed with the exception of 3 of the controls and the group of 4 receiving the lower dose of thyroxine. These remaining animals were sacrificed at 8 weeks.

The growth promoting effect in the thiouracil-fed animal of the 2 compoinds is shown in Figure 2. The weight loss of the control group can be attributed to the combination of thiouracil effect and the suboptimal diet.

TABLE 2. EFFECTS OF *l*-THYROXINE (T-4), TETRAIODOTHYROFORMIC ACID (TFA-4) AND SODIUM IODIDE ON THE EUTHYROID CHOLESTEROL-FED RAT[1]

No. rats	Compound and daily dose (μM/100 gm.)		Thyroid weight (mg./100 gm.)	Serum cholesterol[2] (mg./100 ml.)	Liver cholesterol	
					Conc.[2] (mg./100 gm.)	Total[2] (mg.)
4	CONTROL		6.5 ± 0.9	78 ± 7	1131 ± 245	160 ± 42
5	T-4	0.0039	4.4 ± 0.3	83 ± 7	898 ± 179	116 ± 18
4	TFA-4	2.72	5.2 ± 0.7	84 ± 3	398 ± 82**	48 ± 11**
4	NaI	10.88	6.0 ± 0.4	83 ± 5	1227 ± 131	137 ± 14

[1] Values given are the mean and standard deviation estimated using (n − 1).
[2] Values that differ significantly from the control (t test) are indicated as follows:

* = .01 < P < .05 ** = P < .01

The growth curves of the animals receiving TFA-4, 5.44 and T-4, either 0.0078 or 0.0156 μM/100 gm. are nearly identical. TFA-4 at 2.72 μM/100 gm. resulted in weight gain, but to a lesser degree. The final serum and liver cholesterol levels of these animals are given in Table 3.

Since undetermined amounts of the materials were lost through scattering of food by the animals, the dosage indicated is the maximum ingested.

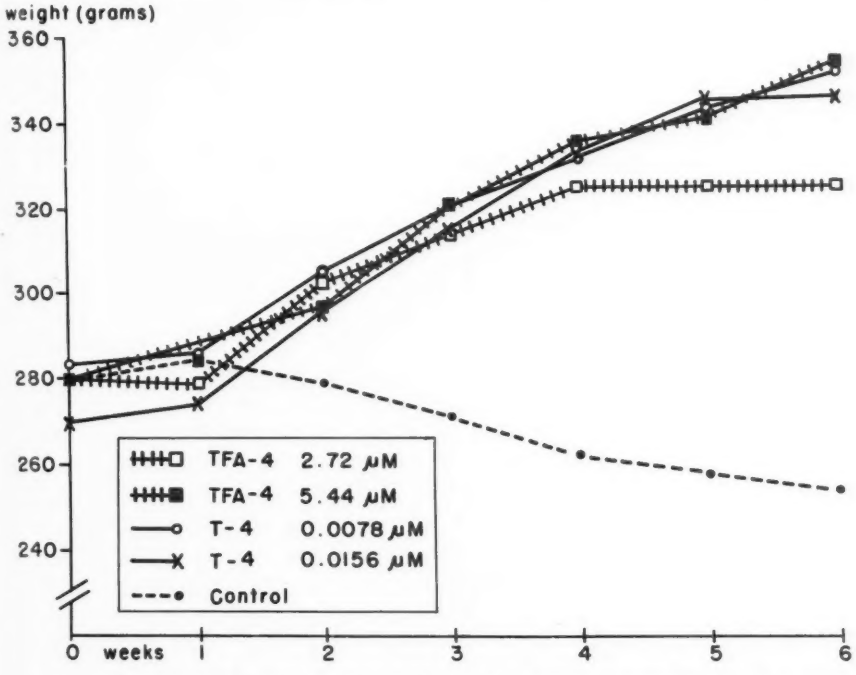

FIG. 2. *Effects of l-thyroxine (T-4) and tetraiodothyroformic acid (TFA-4) on the growth of the cholesterol-thiouracil fed rat.* The compounds were administered orally and the dose indicated (in μM/100 gm. body wt.) is the maximum consumed per day. Final serum and liver cholesterol values of these animals are given in Table 3.

TABLE 3. EFFECTS OF ORAL ADMINISTRATION OF *l*-THYROXINE (T-4) AND TETRAIODOTHYROFORMIC ACID (TFA-4) FOR 6–8 WEEKS[1]

No. rats	Compound and daily dose[2] (μM/100 gm.)	Weight change (gm.)	Thyroid weight (mg./100 gm.)	Serum cholesterol[3] (mg./100 ml.)	Liver cholesterol	
					Conc.[3] (mg./100 gm.)	Total[3] (mg.)
		Six-Week Experimental Period				
8	CONTROL	−26 ± 13	24.9 ± 4.4	198 ± 61	2009 ± 805	195 ± 74
4	T-4 0.0156	+76 ± 12	25.2 ± 4.4	130 ± 19	1548 ± 263	246 ± 55
4	TFA-4 2.72	+44 ± 21	28.1 ± 3.7	115 ± 10*	553 ± 211**	61 ± 22**
4	TFA-4 5.44	+74 ± 8	21.9 ± 3.4	115 ± 16*	395 ± 130**	54 ± 19**
		Eight-Week Experimental Period				
3	CONTROL	−35 ± 3	28.5 ± 3.3	207 ± 27	2220 ± 515	230 ± 55
4	T-4 0.0078	+71 ± 15	26.7 ± 4.9	181 ± 55	2302 ± 496	362 ± 63*

[1] Values given are the mean and standard deviation estimated using (n − 1).
[2] The dose indicated is the maximum possible; due to food scattering somewhat less was actually consumed.
[3] Values that differ significantly from the control (t test) are indicated as follows:

* = .01 < P < .05 ** = P < .01

Effects of TFA-4 on disappearance of cholesterol from serum and liver

Fourteen animals were fed the high cholesterol rabbit chow diet with added thiouracil for a period of 14 days. They were then divided into 3 groups of equal mean weight. Four animals were immediately sacrificed. The remaining 10 animals were maintained for the next 7 days on a diet of rabbit chow, cottonseed oil, and thiouracil, but without added cholesterol.

TFA-4, 2.72 μM/100 gm. body weight, was administered daily subcutaneously to five of the animals. The remaining five received daily subcutaneous saline. At the end of the final week both groups were sacrificed.

When the cholesterol was withdrawn from the diet of the cholesterol-thiouracil fed rat there was an approximate halving of the liver cholesterol concentration during the ensuing 7 days (Table 4). This net reduction in liver cholesterol was significantly greater in the group receiving TFA-4 during the "run-off" period of one week ($P < .01$). Serum cholesterol, on the other hand, was higher in the group receiving TFA-4 than in the saline-injected controls.

TABLE 4. EFFECTS OF TETRAIODOTHYROFORMIC ACID (TFA-4) ON THE DISAPPEARANCE OF CHOLESTEROL FROM SERUM AND LIVER[1]

	No. rats	Thyroid weight (mg./100 gm.)	Serum cholesterol[2] (mg./100 ml.)	Liver cholesterol	
				Conc.[2] (mg./100 gm.)	Total[2] (mg.)
2-week cholesterol-thiouracil diet	4	20.0 ± 2.5	165 ± 31	1088 ± 289	107 ± 19
2-week cholesterol-thiouracil diet followed by 1-week thiouracil diet daily CONTROL injection final week	5	22.9 ± 5.0	92 ± 9	577 ± 156	49 ± 13
2-week cholesterol-thiouracil diet followed by 1-week thiouracil diet TFA-4 2.72 μM/100 gm./day final week	5	25.4 ± 3.9	115 ± 12**	283 ± 23**	30 ± 3*

[1] Values given are the mean and standard deviation estimated using (n − 1).
[2] Values that differ significantly from the control (t test) are indicated as follows:

* = .01 < P < .05 ** = P < .01

DISCUSSION

The biologic activity of TFA-4 in mammals, first reported by Frieden and Winzler (8) who employed the thiouracil goitre inhibition assay, is confirmed by these observations. They determined TFA-4 to have 0.16% the activity of *d*, *l*-thyroxine. This is in close agreement with the present data, by which TFA-4 is calculated to have 0.08% the goitre-preventing activity of T-4, which is responsible for the greater part of the activity of *d*, *l*-thyroxine. TFA-4 was found to be active by the oral route as well as parenterally, although lack of information as to the exact amount taken orally precludes any conclusion as to its relative effectiveness by the two routes.

TFA-3 was also found to be effective in inhibiting thiouracil-induced goitre, its activity being 3 to 4 times that of the 4 iodine analogue. It is of interest that the goitre-preventing activities of TFA-4 and TFA-3 bear a similar relationship to each other as do those of T-4 and T-3.

TFA, having the same basic structure as TFA-4, but lacking the iodine atoms substituted at the 3,5,3′,5′ positions, was without effect on either thiouracil goitre or cholesterol in liver and serum. A similar relationship exists between thyroxine and thyronine, in which hydrogen is substituted for the four iodine atoms of thyroxine (8).

The preliminary report of differences in the physiologic activity of T-4 and TFA-4 is in general confirmed (6). TFA-4, at all levels of administration exerted a relatively greater effect on liver cholesterol than on thiouracil goitre, when compared to T-4.

T-3 and TFA-3 are quite similar with regard to their relative effectiveness in inhibiting thiouracil goitre and in preventing accumulation of cholesterol in the liver. Although at a given level of goitre-inhibition T-3 and TFA-3 both demonstrate somewhat greater effect on liver cholesterol than does T-4, the difference is not marked, and is much less than that observed with TFA-4.

An indication of the probable mechanism by which TFA-4 prevents the accumulation of cholesterol in the liver and serum is provided by the results of its administration to the rat previously "loaded" with cholesterol. Increased biliary excretion of cholesterol and bile acids have been reported to result from the administration of desiccated thyroid to the rat (9). The greater net loss of cholesterol from the livers of the rats receiving TFA-4 as compared to the untreated controls would seem most likely to be due to a similar increased excretion in the bile, either as cholesterol or after conversion to bile acids.

That the effects of TFA-4 on cholesterol metabolism are not dependent upon the hypothyroid state is indicated by its marked effect on liver cholesterol of the euthyroid cholesterol-fed rat. Under the experimental conditions employed, however, it is only in the thiouracil fed rat with its elevated serum cholesterol that a hypocholesterolemic effect of TFA-4 is apparent.

The lack of effect of an equivalent amount of iodine as sodium iodide would indicate that the iodine content of TFA-4 is not in itself responsible for the effect of TFA-4 on cholesterol metabolism.

Whether the other physiologic effects of TFA-4 parallel its goitre-preventing activity or its effect on cholesterol metabolism has been incompletely studied. The data comparing the growth promoting activities of T-4 and TFA-4 with their effects on cholesterol metabolism however suggest that TFA-4 has an exaggerated effect on cholesterol metabolism. At the lower of the 2 doses employed TFA-4 was only partially effective in maintaining growth, the mean weight gain of this group being significantly less than that of the other 3 groups receiving replacement therapy, ($P < .01$). Liver cholesterol in this group was, however, significantly lower than that of the group receiving T-4 at a level twice that necessary to maintain the maximum rate of growth observed ($P < .01$).

Studies in progress indicate that TFA-4 does have an effect on oxygen consumption of the thiouracil-fed rat. Data is insufficient, however, for any quantitative comparison of its calorigenic activity with that of T-4.

The basis for the apparent exaggerated effect of TFA-4 on cholesterol metabolism as compared to its goitre inhibiting and growth promoting effects remains obscure. The possibility that it is due to differences in the activity time courses of T-4 and TFA-4 must be considered. The fact that similar differences between T-4 and TFA-4 were observed in both 2 and 6 week studies lends no support to this hypothesis. Nor does the observation that T-3, with its markedly shorter time activity curve, closely resembles T-4 in its relative effect on cholesterol metabolism and thiouracil-induced goitre.

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PARATHYROID AND GONAD RELATIONSHIP IN REGULATING BLOOD CALCIUM FRACTIONS IN CHICKENS[1,2]

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ABSTRACT

Plasma diffusible calcium levels of cocks and capons with or without estrogen treatment are significantly decreased by parathyroidectomy. The level of non-diffusible calcium is increased by estrogen in sham and starved controls, but not when estrogenized birds are parathyroidectomized and diffusible levels are significantly decreased. Spontaneous recovery from parathyroidectomy occurs in about 6 days. Cocks and capons injected with estrogen after parathyroidectomy show no increase in non-diffusible levels when diffusible levels are still abnormally low by 4 days after the operation. Parathyroids and estrogen are suggested to be indirectly related in regulating plasma calcium of birds. The parathyroids maintain diffusible calcium at normal levels so that estrogen can increase non-diffusible calcium.

ESTROGEN and substances estrogen-like in action increased blood calcium levels of cocks (1), immature pullets (2), cocks and drakes (3), pigeons (4, 5), sparrows (6), quail (7) and other birds. The rise in blood calcium occurred only in the non-diffusible calcium (8, 9), which was the fraction investigators (10, 11, 12) had found much higher in laying than in non-laying birds.

Total calcium levels were markedly decreased by removal of the parathyroids from non-estrogenized ducks (13) and pigeons (8, 9). Estrogen administered to ducks before or soon after parathyroidectomy did not increase these low blood calcium levels (13). Furthermore, ducks that were pretreated with estrogen to elevate blood calcium to levels of approximately 30 mg./100 ml. had levels of 19.0 mg./100 ml. soon after extirpation of the parathyroids and died in tetany (14). Laying hens that were parathyroidectomized show a marked loss in blood calcium (15), substantiating the results obtained with estrogen-treated ducks. On the other hand, parathyroidectomized pigeons maintained on $CaCl_2$, dihydrotachysterol, and/or $Al(OH)_3$, showed an increase in blood calcium when estrogen treated (8, 16).

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Investigators studying the relationship between estrogen and parathyroid hormone in the duck suggested that estrogen required the presence of the parathyroid hormone to augment blood calcium, but those using pigeons reported that estrogen increases total calcium levels in the absence of the parathyroid hormone.

A suggestion was recently made, on the basis of studies with parathyroidectomized laying hens, that when diffusible calcium levels are abnormally low, non-diffusible calcium levels are not increased by estrogen (15). Supporting data are presented in this paper.

METHODS

White Leghorn male and gonadectomized male chickens, 4 to 8 months of age were used in these experiments. The cocks and capons were maintained in batteries and fed a commercial mash, *ad libitum*. Cocks were gonadectomized at 8 weeks of age. Methods for handling blood samples, for determining calcium and for removal of the parathyroids have been described elsewhere (15, 17). High calcium concentrations in plasma were analyzed in 0.25 ml. samples rather than 0.5 ml. samples, as previously described (17). Estradiol, in 95% alcohol, was administered into the pectoral muscles at 2 mg. per injection to obtain augmented blood calcium levels in a short response time. Alcohol had no influence on blood calcium levels.

RESULTS

Total plasma calcium averaged 11.08 and 11.59 mg./100 ml. for cocks and capons respectively and ranged from 10.0 to 13.5 mg./100 ml. Diffusible calcium ranged from 4.0 to 9.0 mg./100 ml. and averaged 6.21 and 6.24 mg./100 ml. for the same groups (Table 1).

Plasma total calcium of cocks and capons starved for 24 hours did not change appreciably but there was a decrease in the non-diffusible fraction and an increase in the diffusible fraction (Table 1). Forty-eight hours of starvation produced a further divergence of these calcium fractions in the cock; diffusible levels increased 13% over control values, while non-diffusible levels decreased 18%.

Parathyroidectomy of cocks and capons. The mean values for both plasma calcium fractions of cocks and capons were significantly below levels of sham operated birds at 18–24 hours after parathyroidectomy (Table 2). The response to parathyroidectomy was quite variable. Some chickens showed a marked reduction in both plasma calcium fractions while others showed no response at all. Total calcium levels were markedly reduced only in birds having abnormally low diffusible calcium levels, and symptoms of hypoparathyroidism, as previously described (18, 19) were observed only in those birds with a marked decrease in plasma calcium. Death rarely followed removal of the parathyroids and recovery occurred spontaneously in 1 to 10 days, usually by the 6th day.

The decrease in diffusible calcium accounted for 52.1 and 63.1% of the loss in total calcium levels of cocks and capons respectively (Table 2).

TABLE 1. PLASMA CALCIUM FRACTIONS IN FED AND 24-HOUR STARVED COCKS AND CAPONS

Calcium—mg./100 ml.	Cocks			Capons		
	Normal	24-hour starvation	Diff.	Normal	24-hour starvation	Diff.
Diffusible	(77)* 6.21	(39) 6.47	+0.26	(9) 6.24	(11) 6.48	+0.24
Non-diffusible	(77) 4.87	(39) 4.50	−0.37	(9) 5.35	(11) 4.44	−0.91
Total	(77) 11.08	(39) 10.97	−0.11	(9) 11.59	(11) 10.92	−0.67

* ( ) = No. of birds.

Analysis of variance of diffusible and non-diffusible calcium data

Source of variation	*d.f.*	*Mean square*
Sex	1	.31
Calcium	1	163.38**
Treatment	1	.45
Sex × Calcium	1	.00
Sex × Treatment	1	.84
Calcium × Treatment	1	6.46*
Sex × Calcium × Treatment	1	1.74
Remainder	264	1.34

* Significant at P = .02.
** Significant at P < .01.

Parathyroidectomy of cocks and capons pretreated with estrogen. Forty-eight hours after the first of 2 estrogen injections, administered 24 hours apart, all cocks and capons had calcium levels characteristic of those in laying hens, *i.e.* in the range of 16.0 to 30.0 mg./100 ml. At this time, the chickens were again administered estrogen and either starved for 24 hours, sham parathyroidectomized or parathyroidectomized. Total calcium levels

TABLE 2. PLASMA CALCIUM FRACTIONS 18–24 HOURS AFTER SHAM PARATHYROIDECTOMY (SP) AND PARATHYROIDECTOMY (P) OF COCKS AND CAPONS 4–8 MONTHS OF AGE

Calcium—mg./100 ml.	Cocks			Capons		
	SP	P	Diff.	SP	P	Diff.
Diffusible	(23)* 6.43	(25) 4.59	−1.84	(20) 6.88	(20) 5.19	−1.69
Non-diffusible	(23) 5.55	(25) 3.88	−1.67	(20) 5.02	(20) 4.03	−0.99
Total	(23) 11.98	(25) 8.47	−3.51	(20) 11.90	(20) 9.22	−2.68

* ( ) = No. of birds.

Analysis of variance of diffusible and non-diffusible calcium data

Source of variation	*d.f.*	*Mean square*
Sex	1	1.80
Calcium	1	54.79**
Operation	1	108.37**
Sex × Calcium	1	5.62*
Sex × Operation	1	1.98
Calcium × Operation	1	1.98
Sex × Calcium × Operation	1	.01
Remainder	168	1.24

* Significant at P ≅ .02.
** Significant at P < .01.

of the starved (20 birds) or sham operated cocks and capons (28 birds) continued to increase significantly ($P = .02$); whereas the 35 birds that were parathyroidectomized showed a small but significant decrease in total levels (Figure 1). The diffusible calcium fraction of these capons and cocks pretreated with estrogen and then parathyroidectomized decreased to 58.1% of the pre-operative level. These diffusible levels were similar to those in parathyroidectomized birds not estrogen-treated as shown in Table 2. The non-diffusible fraction of the capon and cock, pre-treated with estrogen, was lowered, but not appreciably by extirpation of the para-

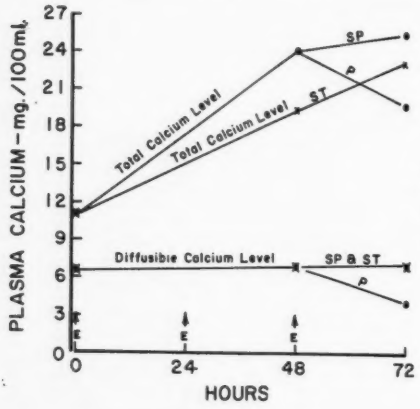

FIG. 1. Plasma total and diffusible calcium levels of cocks and capons treated with estrogen (E) and then starved (ST), sham parathyroidectomized (SP) or parathyroidectomized (P) 48 hours after the 1st estrogen injection.

thyroids. However, in the sham operated or starved birds, the non-diffusible level continued to increase as a result of the estrogen treatment and accounted for the significant increase observed in the total calcium level (Fig. 1).

Parathyroidectomy of cocks and capons followed by estrogen administration. Cocks and capons were parathyroidectomized and then treated with estrogen for 3 days after the operation. On the 4th day, the mean total calcium level of these chickens was 20.12 mg./100 ml. (Table 3). Diffusible calcium levels averaged 4.63 mg./100 ml. The latter figure was higher than that for birds immediately following parathyroidectomy and indicated that these birds were recovering from the operation. The gradual return of diffusible calcium levels toward normal, and the lessening of hypoparathyroidal symptoms were presumably due to the elaboration of parathyroid hormone by accessory parathyroid tissue, which is found in chickens (18, 19). However, not all of the parathyroidectomized birds, by the 4th day after the operation, showed the response to estrogen treatment. Those which still had the abnormally low diffusible calcium levels were the ones which showed no response (Table 3). Thus, the degree of response to estrogen was related to the extent of recovery from parathyroidectomy and the level of diffusible calcium was a measure of the recovery.

DISCUSSION

The blood calcium response of the chicken to parathyroidectomy is extremely variable and large numbers of birds per treatment must be used to determine whether effects are significant. This variability in response and the recovery observed in parathyroidectomized chickens are presumably related to the presence of accessory parathyroid tissue. This is true of laying hens (15) as well as cocks and capons.

The parathyroid hormone in chickens appears to be concerned primarily with the regulation of the diffusible calcium fraction rather than the non-

TABLE 3. PLASMA CALCIUM FRACTIONS IN COCKS AND CAPONS PARATHYROIDECTOMIZED THEN TREATED WITH ESTROGEN FOR 3 DAYS

Blood samples were removed on the 4th day after the operation. These data are arranged to show the relationship between diffusible calcium levels and non-diffusible calcium levels

Diffusible calcium, mg./100 ml.	Sex	Observed values—mg./100 ml.		
		Total calcium	Non-diffusible calcium	Diffusible calcium
5.0–	Capon (2)*	22.45	17.05	5.40
	Cock (7)	22.82	16.92	5.90
4.6–4.9	Capon (2)	24.20	19.40	4.80
	Cock (1)	27.50	22.90	4.60
4.1–4.5	Capon (1)	24.30	20.20	4.10
	Cock (1)	21.90	17.50	4.40
3.6–4.0	Capon (2)	17.40	13.50	3.90
	Cock (1)	22.60	18.60	4.00
–3.5	Capon (3)	8.70	5.53	3.17
	Cock (1)	7.40	4.70	2.70
	(21)	20.12	15.49	4.63

* ( ) = No. of birds.

diffusible fraction. Diffusible levels are significantly decreased upon removal of the parathyroids from cocks and capons, with or without estrogen treatment. Also, the non-diffusible fraction accounted for less of the total loss in plasma calcium, and much of the decrease in this fraction may be due to starvation. Furthermore, the non-diffusible calcium level, excluding the effect by starvation, decreased in parathyroidectomized hens only when the diffusible calcium levels were significantly decreased (15). A similar effect was noted in the cocks and capons not estrogen-treated.

The ability of estrogen to augment plasma non-diffusible calcium appears to be dependent on the action of parathyroid hormone to regulate diffusible calcium levels. Thus, an abnormally low diffusible calcium level following parathyroidectomy resulted in the failure of estrogen to increase or maintain non-diffusible calcium levels. A level significantly lower than normal but not abnormally low altered the extent of the increase in bound calcium caused by estrogen. Ducks are particularly sensitive to removal of the parathyroids and estrogen does not maintain or increase total calcium levels in these operated birds (13, 14). Thus the data on chickens

with abnormally low diffusible levels agrees with the data on ducks. Riddle and collaborators (8, 9) stated that total calcium was increased by estrogen in pigeons independent of the parathyroid hormone; however, their data are based on results in parathyroidectomized pigeons maintained with normal diffusible calcium levels by chemical treatment. Some of their data (9) showed estrogenized pigeons dying in tetany with abnormally low diffusible calcium levels and low non-diffusible calcium levels. Chickens which show little or no decrease in diffusible calcium levels after parathyroidectomy also show the estrogenic effect on bound calcium levels. Thus, the data on chickens, ducks and pigeons are essentially alike and, according to our interpretation, indicate a relationship, though indirect, between parathyroid hormone and estrogen.

These data suggest that parathyroid hormone and estrogen are indirectly related in their action on blood calcium. Parathyroid hormone maintains diffusible calcium levels so that estrogens can increase non-diffusible calcium levels.

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DISTRIBUTION OF 3:5:3′-TRIIODOTHYROACETIC ACID IN THE RAT[1]

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ABSTRACT

Studies with triiodothyroacetic acid (triac) in myxedema indicate, though similar in metabolic effect, it is less potent than triiodothyronine. Similar results have been obtained when *in vitro* oxygen consumption of tissues was measured following *in vivo* administration of these compounds. It is possible that the weak metabolic effect of *exogenous* triac is due to failure of this compound to enter cells.

Tissue concentrations of triac, triiodothyronine and thyroxine were compared following intravenous administration of tracer (0.01 μg.) quantities of these I^{131}-labelled compounds. Concentrations in plasma, muscle, heart, liver, small intestine and kidney were measured.

Thyroxine remained largely within the vascular compartment. Triiodothyronine very rapidly left the intravascular compartment and reached relatively high concentrations in kidney, liver, heart and muscle. The concentration of triac in plasma fell rapidly due to clearance by the liver and excretion into the small intestine. Triac did not accumulate in muscle, heart or kidney. The radioactivity in tissues following triiodothyronine was identified as the injected compound. Following triac, appreciable amounts of iodide appeared.

It is suggested that conclusions regarding comparative metabolic activity of these compounds administered *in vivo* should be interpreted on the basis of their extent of incorporation into the cell.

THE maximal metabolic effects of thyroxine occur after a latent period of several days following administration of this hormone. It has been suggested that this delay in response is due to transformation in the hormone which occurs in the peripheral tissues. Consistent with this hypothesis is the observation that *l*-3:5:3′-triiodothyronine (hereafter referred to as triiodothyronine) given to the intact animal exerts a more rapid action than *l*-thyroxine. The deiodination of thyroxine to triiodothyronine may be one step in the transformation of the thyroid hormone into a form active at the cellular level. Experimental data have been presented to show that this reaction does take place; for example, *in vitro* systems using rat kidney slices (1, 2) and *in vivo* both in the propyl-thiouracil treated rat (3) and in the athyreotic human (4).

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The administration of triiodothyronine to the intact animal is still attended by a latent period, and this hormone shows no immediate effect on oxygen uptake when added to tissue slice preparations (5). Therefore, it is logical to search beyond triiodothyronine for further transformations involved in the derivation of thyroid hormone(s) active at the intracellular level. When labelled triiodothyronine is added to *in vitro* systems containing homogenate or mitochondria of rat kidney and liver, labelled 3:5:3′-triiodothyroacetic acid (hereafter referred to as triiodothyroacetic acid) can be identified (6, 7). Labelled triiodothyroacetic acid has also been recovered from rat kidney and muscle after injection of labelled triiodothyronine into the intact animal (8, 9). The conversion of triiodothyronine to its acetic acid analogue may represent a purely degradative process, but the possibility that triiodothyroacetic acid has physiologic activity at the cellular level is equally plausible.

The physiologic effects of triiodothyroacetic acid have been studied *in vitro*. Thibault (10) reported stimulation of oxygen consumption within 15 minutes when triiodothyroacetic acid was added to rat kidney slices. Wang and Lardy (11) have found that triiodothyroacetic acid is effective in uncoupling oxidative phosphorylation in heart mitochondria at about 1/10 the concentration required for triiodothyronine. It has been suggested that, because of its rapid and potent action *in vitro*, triiodothyroacetic acid may be a cellularly active form of thyroid hormone. *In vivo* studies with triiodothyroacetic acid (12, 13, 14, 15) indicate that its effects in human myxedema are qualitatively similar to those of triiodothyronine, although considerably less potent. A similar conclusion regarding relative potency of triiodothyroacetic acid and triiodothyronine was reached by Barker (16), who compared the effect of pharmacologic doses of thyroxine, triiodothyronine, tetraiodothyroacetic acid and triiodothyroacetic acid administered to rats for four days pre-mortem on oxygen consumption of surviving tissue slices. With this system he found that although active, triiodothyroacetic acid had less metabolic effect than triiodothyronine. These studies could be interpreted as evidence against the hypothesis that triiodothyroacetic acid may be the active form of the thryoid hormone in the tissues. On the other hand, such a conclusion is not justified until the metabolic fate of triiodothyroacetic acid is compared to that of thyroxine and triiodothyronine following administration of equal quantities of these compounds. It is possible that the relatively weak effect of *exogenous* triiodothyroacetic acid observed in human and animal studies results from failure of this compound to reach comparable concentrations within the cells.

The present experiment was designed to compare the tissue concentration of triiodothyroacetic acid, triiodothyronine and thyroxine following administration of tracer quantities of these substances.

METHODS

Male rats of the Sprague-Dawley strain weighing 180–200 grams were used in all experiments. The animals were kept on a standard commercial laboratory chow prior to and during the period of experiment. In the distribution study the test compounds,[2] originally dissolved in 50% propylene glycol, were diluted in saline to contain 0.02 μg. per ml. One half milliliter of this dilution (0.01 μg.) was injected into the tail vein. At the indicated time intervals the rats were anesthetized with ether and exsanguinated by cardiac puncture using a heparinized syringe. Ordinarily, from 6 to 8 ml. of blood were withdrawn. The organs were removed, thoroughly washed with running water, and blotted dry before weighing. In the case of heart and kidneys, entire organs were used. Portions of the liver and the skeletal muscle of the abdominal wall, each approximating 3 gm., were used. Nearly all of the small intestine, with contents, beginning just distal to the pylorus approximating 3 gm., was removed. All of the tissues were weighed with a Roller-Smith torsion balance, placed into test tubes and counted in a well-type scintillation detector. The plasma was separated by centrifugation and 3 ml. counted.

A dilution of the injected material was counted daily to permit correction for isotopic decay and counter variability, and to serve as a standard for calculation of per cent uptake. The values given represent the average of at least four animals per time point. Since all animals weighed between 180 and 200 gm., a correction for total weight of animal was not necessary.

In separate experiments designed to identify the radioactivity in the tissues following administration of labelled triiodothyronine and triiodothyroacetic acid, it was necessary to give one microgram of each compound per rat. This was given subcutaneously in 50% propylene glycol. At intervals of 1-3-6-12 and 24 hours following injection the rats were killed and blood and organs removed as described above. The tissues were homogenized in 3 ml. water and extracted with 10 volumes of 3% NH_4OH in butanol. The butanol extracts were concentrated under reduced pressure and applied to Whatman #3 MM paper for chromatography. Carrier triiodothyroacetic acid[3] and triiodothyronine[4] were added for identification. The solvent system used for separation was tertiary amyl alcohol saturated with 2N NH_4OH(7). Radioactivity was located on the strip with a chromatograph scanner, and the position of the known carrier determined by color development with 4-amino-antipyrine (17).

In experiments designed to check excretion of radioactivity following labelled triiodothyronine and triiodothyroacetic acid administration, 0.1 μg. of each compound in 50% propylene glycol was given subcutaneously. Urine and feces were collected separately for 24, 48 and 72 hours following injection. The entire fecal sample was counted, using the well-type scintillation detector. Each urine sample was diluted to a standard volume, and an aliquot was counted. The excretion in urine and feces was calculated as per cent of of administered radioactivity corrected for decay. Identification of the radioactivity in the urine and feces was accomplished by chromatography. An appropriate volume of urine was extracted with 10 volumes of ammoniacal butanol. From 5 to 10 gm. of feces were air dried, finely powdered and extracted with 200–400 ml. of ammoniacal butanol. The butanol extracts were chromatographed as described above.

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[2] I^{131}-labelled chromatographically pure *l*-thyroxine, *l*-3:5:3′-triiodothyronine and 3:5:3′-triiodothyroacetic acid were obtained from Abbott Laboratories.

[3] Obtained from Hoffmann-La Roche, Inc. through the courtesy of M. J. Schiffrin.

[4] Obtained from Smith, Kline and French Laboratories through the courtesy of A. E. Heming.

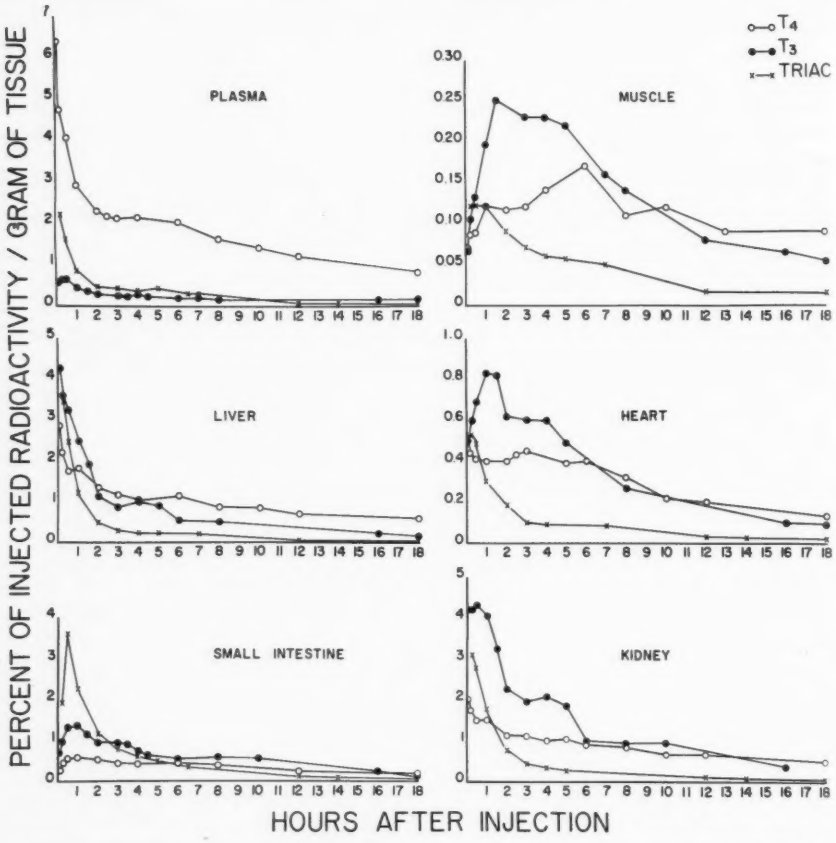

FIG. 1. Distribution of radioactivity within the tissues following injection of I^{131}-labelled triiodothyroacetic acid, triiodothyronine and thyroxine.

x—x triiodothyroacetic acid
●—● triiodothyronine
○—○ thyroxine

RESULTS

Distribution of Radioactivity in Tissues

The results of these experiments are shown in Figure 1.

Plasma: It will be noted that both triiodothyronine and triiodothyroacetic acid are rapidly cleared from plasma. Triiodothyronine leaves the vascular compartment so rapidly that even at 6 minutes only one-tenth as much remains as compared with thyroxine. Triiodothyroacetic, though higher than triiodothyronine at the earliest time points, leaves the plasma rapidly so that by 10 hours its concentration is lower than triiodothyronine. The plasma level of thyroxine remains comparatively high throughout the period of observation.

Liver: Both triiodothyronine and triiodothyroacetic acid appear initially in higher concentration than thyroxine. However, they fall rapidly and from two hours on are lower than thyroxine. Triiodothyroacetic acid is cleared much more rapidly from the liver than either triiodothyronine or thyroxine, and by 12 hours has almost disappeared.

Small Intestine: Triiodothyroacetic acid rapidly appears in the intestine reaching maximum concentration in 30 minutes, at which time its concentration is seven times as great as thyroxine and three times as great as triiodothyronine. After this, it falls rapidly so that by 6 hours its concentration is lower than either triiodothyronine or thyroxine.

Muscle: Of the three compounds, triiodothyronine enters the muscle most rapidly, reaching a maximum concentration between 1 and 2 hours and falling gradually. Thyroxine enters muscle slowly, attaining peak concentration at 6 hours and decreases slowly so that after 10 hours its concentration exceeds that of triiodothyronine. In contrast, triiodothyroacetic acid attains its maximum concentration in 30 minutes and rapidly decreases after 1 hour so that at 12 hours its concentration is one-third to one-fifth that of triiodothyronine and thyroxine.

Heart: The relative concentrations of the three compounds in heart muscle is similar to that observed in skeletal muscle; i.e., an initial rapid triiodothyronine peak with gradual decrease, a delayed rise in thyroxine with slower decrease, and with triiodothyroacetic acid a maximum peak at 15 minutes with rapid disappearance.

Kidney: As with the liver, the initial concentration of triiodothyronine and triiodothyroacetic acid exceeds that of thyroxine, but triiodothyroacetic acid concentration falls very rapidly so that by 2 hours it is lower than thyroxine and is negligible by 12 hours. The concentration of triiodothyronine remains higher than triiodothyroacetic acid, but falls more rapidly than thyroxine so that by 18 hours it is less than thyroxine.

Identification of Radioactivity in Tissues

In the case of triiodothyronine most of the radioactivity present in all tissues was identified as the injected compound. Small quantities of unidentified radioactivity with an R_f value characteristic of iodide appeared early and increased slightly during the experiment. With triiodothyroacetic acid, though at 1 and 3 hours most of the radioactivity could be identified as triiodothyroacetic acid, at 12 and 24 hours most of the radioactivity was present as iodide. Radioactivity in muscle failed to reach levels high enough to permit chromatographic identification. The results of these experiments are shown in Figure 2.

Excretion Study

As seen in Table 1 and Figure 3, triiodothyroacetic acid is excreted largely unchanged in the feces. However, approximately 19% is deiodinated and the iodide excreted in the urine. Triiodothyronine, on the other

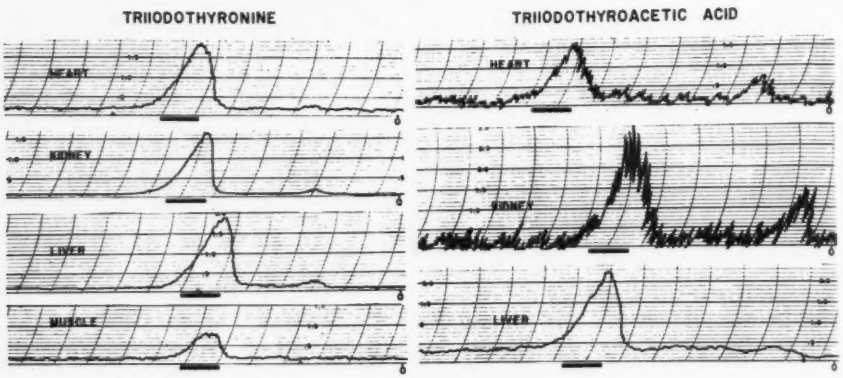

FIG. 2. Identification of labelled triiodothyroacetic acid and triiodothyronine in tissues 3 hours following injection. Position of non-radioactive carrier shown by bar. O—origin of chromatogram. Chromatographic front not shown. Peak of radioactivity close to origin has an R_f value characteristic of iodine.

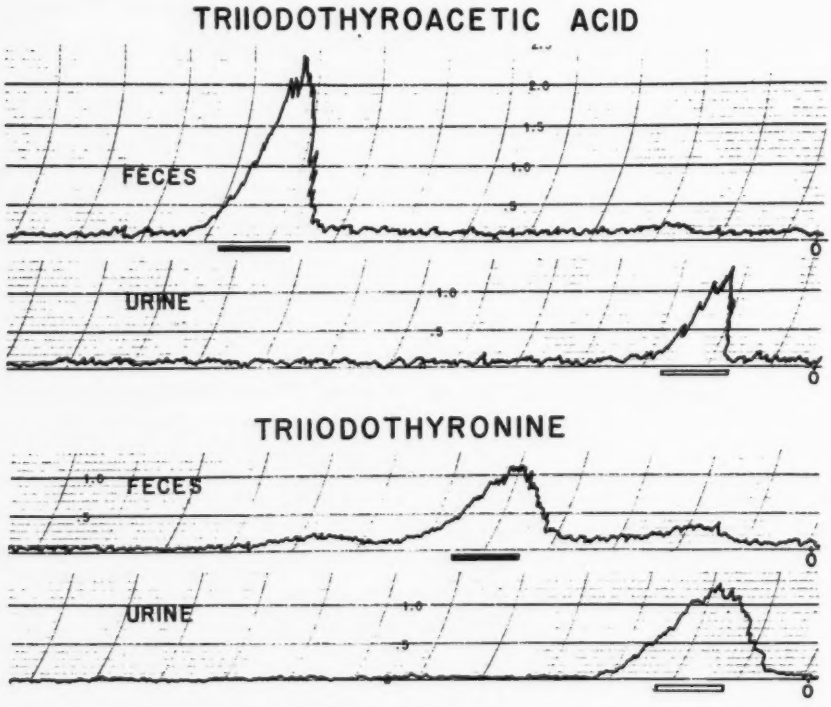

FIG. 3. Identification of radioactivity in feces and urine following injection of I^{131} labelled triiodothyroacetic acid and triiodothyronine. Position of non-radioactive carrier shown by solid bar. The open bar indicates the position of iodide. O-origin of chromatogram. Chromatographic front not shown.

hand, is deiodinated to a greater extent, as 55% of the radioactivity appears as iodide in the urine.

DISCUSSION

Three major conclusions may be drawn from these results. *One*, thyroxine following injection remains largely within the intravascular compartment. *Two*, triiodothyronine very rapidly leaves the intravascular compartment and reaches relatively high levels in the kidney, liver, heart and skeletal muscle. *Three*, triiodothyroacetic acid, despite its rapid clearance from plasma, does not accumulate in heart, skeletal muscle or kidney.

The retention of thyroxine within the intravascular compartment can be explained by the presence in plasma of a specific thyroxine binding protein (TBP). Triiodothyronine, having less affinity for the TBP than thyroxine, is not held within the intravascular compartment. For this reason and because it enters the large muscle mass, kidney and liver, its concentration in

TABLE 1. EXCRETION OF RADIOACTIVITY IN URINE AND FECES COLLECTED DURING THE INTERVALS 0–24 HRS., 24–48 HRS., AND 48–72 HRS. AFTER ADMINISTRATION OF LABELLED TRIIODOTHYRONINE AND TRIIODOTHYROACETIC ACID

Note that the per cent of radioactivity was measured on passed feces and does not closely reflect the amount excreted into the small intestine during the same interval.

	Triiodothyronine		Triiodothyroacetic acid	
	Urine	Feces	Urine	Feces
0–24 hr.	32.1%	26.8%	13.7%	34.4%
24–48 hr.	19.6%	13.7%	5.0%	38.7%
48–72 hr.	3.8%	1.5%	0.3%	4.9%

plasma falls rapidly. Triiodothyroacetic acid, despite a weaker affinity than triiodothyronine for TBP, leaves plasma less rapidly, due, probably to its failure to enter muscle and kidney to a comparable extent. This difference in volume of distribution is in agreement with the findings of Rall (15) who reported a greater triiodothyronine than triiodothyroacetic acid volume in the athyreotic human subject. The fairly rapid clearance of triiodothyroacetic acid from plasma can be accounted for by its rapid elimination by the liver into the small intestine.

The results observed in this experiment might provide an interpretation for the relatively weak metabolic effect of administered *exogenous* triiodothyroacetic acid observed in human or animal studies. This compound does not enter cells, specifically heart, kidney and skeletal muscle, to the same extent as triiodothyronine or thyroxine, and is simultaneously rapidly eliminated by the liver. Penetration into the cell, or some compartment within the cell, is an essential prerequisite to the action of a hormone. Thus, if triiodothyroacetic acid is a metabolically active derivative of thyroid hormone formed within the cell, mechanisms for transporting it across cell membranes may not be present.

Acknowledgment

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FURTHER STUDIES ON THE BLOOD GLUCOSE AND PANCREATIC ISLETS OF LIZARDS[1]

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ABSTRACT

In this investigation, the changes in the blood sugar and the pancreatic islet cells of a lizard, *Eumeces obsoletus*, were studied after the administration of adrenaline, glucagon, hydrocortisone, cobaltous chloride, and Synthalin A and after hypophysectomy and pancreatectomy.

Adrenaline, glucagon, and hydrocortisone all produced hyperglycemia and beta cell degranulation. The blood sugar response was the greatest with glucagon while the beta cells were the most stimulated as a result of hydrocortisone hyperglycemia. Cobaltous chloride produced a hyperglycemia but no observable histological changes. Synthalin A evoked a severe hypoglycemia accompanied by alpha cell degranulation together with a small degree of alpha cell necrosis. Hypophysectomy produced a moderate hypoglycemia while total pancreatectomy resulted in a severe hypoglycemia. Insulin given to pancreatectomized animals resulted in a moderately high hyperglycemia.

From these and previous studies on the lizard, the pancreatic beta cell, as evidenced by its destruction with alloxan and subsequent hyperglycemia and its stimulation and degranulation by glucose, adrenaline, and hydrocortisone, is undoubtedly the site of origin of insulin. The production of a blood glucose maintaining and elevating factor by the pancreas of lizards is indicated by the observations that these animals are very glucagon sensitive and that in the absence of the pancreas, they become severely hypoglycemic. The latter fact would tend to indicate that lizards, like certain birds are more dependent on a pancreatic factor for the maintenance of the blood sugar level than are other vertebrates which so far have been investigated. We interpret the marked hyperglycemia evoked by insulin in the pancreatectomized animal as a response to the hyperglycemic-glycogenolytic factor present in certain mammalian insulin preparations acting in an unapposed situation (no lizard beta cells or insulin). In addition, it may be that the lizard is more responsive to the blood sugar raising substance (glucagon) of mammalian preparations than it is to the blood sugar lowering material (insulin). Thus, the hypoglycemic action of insulin may be masked and the animal made to appear "insulin resistant."

Although lizards become hypoglycemic after administration of Synthalin A, the failure to destroy selectively the alpha cells leaves undecided the question of the source of the hyperglycemic factor. However, indirect evidence, such as the stimulation of the alpha cell after chronic insulin administration and by starvation would implicate it as a likely source of such a material.

IN an earlier work (1) we suggested that the insulin resistance of lizards may be correlated with the relatively large amount of alpha cell tissue in

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[1] This investigation was supported by a United States Public Health Service Grant (A-1192).

[2] A portion of the material in this paper is from the doctoral thesis of the junior author.

the pancreas which might be producing a hyperglycemic or fat mobilizing substance. The present study is an extension of the earlier work, with the primary object being to establish the role of the alpha cell in the maintenance of the blood glucose level. The blood glucose and the changes in the pancreatic islet cells were studied after treatment with adrenaline, glucagon, hydrocortisone, cobaltous chloride, and Synthalin A, and after hypophysectomy and pancreatectomy. In a previous publication (1) the effects of glucose, insulin, and alloxan were reported.

MATERIALS AND METHODS

Freshly captured specimens of a skink, *Eumeces obsoletus*, were used in this investigation. All animals were fasted at least seven days before study and were kept in cages in a room at 70° F. Blood samples were obtained from the carotids after decapitation and the blood glucose determined by averaging the values of duplicate or triplicate specimens from each animal. True blood glucose values were determined by the Somogyi copper-iodometric method (3). For routine study the pancreases were excised and fixed in Bouin's solution and 5μ paraffine sections were stained with Gomori's chromalum hematoxylin and phloxine method (4). To determine the argyrophilia of islet cells both formalin and Bouin fixed tissues were stained with the Bielschowski-Gros, Glees-Meyer, and Bodian's protargol methods. Adrenaline, glucagon, cobaltous chloride, Synthalin A, and hydrocortisone were administered in the manner and dosages indicated in Table 1.

Hypophysectomy was performed on Nembutal anesthetized animals. After entering the oral cavity through a neck incision just lateral to the trachea, a hole was drilled with a dental burr in the basisphenoid bone just underlying the sella turcica and the pituitary gland exposed. After visualization the gland was aspirated through a glass cannula.

Pancreatectomy was performed on Nembutal anesthetized animals. After opening the abdominal cavity through a lower left abdominal incision, the dorsal mesentery containing the pancreas was pulled ventrally. Since the right pancreatic lobe intimately encircles the common bile duct it is necessary to ligate the duct as close to its exit from the liver as possible. The larger arteries supplying the spleen and pancreas were then ligated. As it has been histologically observed that a small part of the pancreas may be incorporated between the outer longitudinal and inner circular muscular layers of the duodenum, in most animals a section of the duodenum containing the entering pancreatic and bile ducts was resected and the individual cut ends of the gut sutured closed. The pancreas and spleen were then removed. The spleen was always removed with the pancreas, as the splenic end of the pancreas is frequently closely attached to or actually incorporated in the splenic tissue. Since such a radical procedure is necessary to completely pancreatectomize a lizard, it is not feasible to permit them to live more than 2 or 3 days after operation. After hypophysectomy the ability of animals to withstand pancreatectomy is greatly reduced, and only 2 out of 12 animals subjected to a combined operation recovered from the anesthetic and were active 24 hours after operation.

OBSERVATIONS

Observations on blood glucose

In an earlier study (1) the normal blood glucose of *Eumeces obsoletus* after fasting was determined to be 112 mg.%, after oral glucose 179 mg.%, and after alloxanization 168 mg.%. After insulin administration there was a very short period of hypoglycemia followed by a mild compensatory

hyperglycemia or a return to near normal levels (for details refer to Table 1).

After adrenaline, an average hyperglycemia of 166 mg.% was evoked. Cortisone (20 mg./kg. given to 5 animals daily for 3 days) produced no rise in blood sugar. Hydrocortisone, on the other hand, produced an average

TABLE 1. CHANGES IN THE BLOOD SUGAR AND PANCREATIC ISLETS OF THE LIZARD, *EUMECES OBSOLETUS*, AFTER VARIOUS TREATMENTS

Treatment or condition	No. of animals	True blood glucose in mg.%		Histologic condition of the pancreatic islets
		Average	Range	
1. Normal, untreated	10	112	91–150	alpha and beta cells with varying degrees of granulation
2. Post-absorptive (1–4 days)	10	179	141–224	as in 1
3. Glucose, oral (8 mg./kg.; sacrificed 2 hrs. after administr.)	3	208	153–247	beta cell degranulation
4. Adrenalin (0.2–0.4 ml., 1:100,000 i.p.; sacrificed 15–40 minutes after injection)	6	166	89–209	beta cell degranulation
5. Glucagon (Lilly, Lot #258-234B-40) (0.2 mg./kg.; sacrificed 1 hr. after injection)	6	235	183–264	beta cell degranulation
6. Hydrocortisone (20 mg./kg. i.p. every other day ×3, sacrificed 7th day after first injection)	5	156	135–189	beta cell hypertrophy and degranulation
7. Alloxan				
a. 300 mg./kg. i.p. every other day ×4; sacr. 8th day after 1st injection. (Total 1.2 gm.)	5	112	70–164	beta cell degranulation
b. 300 mg./kg. i.p. every other day ×5; sacr. 14th day after 1st injection (Total 1.5 gm.)	7	168	146–209	beta cell destruction
8. Insulin (Crystalline Zn., Lilly)				
a. (3000 u. cryst. Zn. Insulin/kg. i.p.)				
(1) sacr. after 10 min.	2	34	34	alpha cell degranulation
(2) sacr. after 20 min.	2	31	28– 34	
(3) sacr. after 30 min.	2	82	80– 84	
(4) sacr. after 60 min.	3	111	100–113	
(5) sacr. after 120 min.	2	130	130	
(6) sacr. after 21 hours	4	70	45–100	
b. (2000 u./kg. every day ×4, sacrificed 60 min. after 4th injection)	3	112	80–142	degranulated alpha cells with darker than normal nuclei; small inactive beta cells
9. Synthalin A				
a. 6 mg./kg. i.p., sacr. 24 hrs. after 1 injection	4	105	57–147	no effect
b. 6 mg./kg., i.p., every day ×3, sacr. 4th day after first injection	8	less than 8	less than 8	degranulation of alpha cells (no marked toxic effects)
10. Cobaltous Chloride (10 mg./kg. i.p. daily ×4; sacrificed 5th day after 1st injection)	15	150	95–233	normal variation
11. Cobaltous chloride and Synthalin A. (Same dosages as in 9 and 10, daily ×4, sacrificed 5th day after 1st injn.)	4	3 animals less than 8, 1 animal 24		same as 9
12. Hypophysectomized (sacr. 2–10 days after operation)	16	82	8–128	mild increase in alpha and beta cell degranulation
13. Pancreatectomized	11	36	8– 88	
14. Hypophysectomized (day 1); Pancreatectomized (day 3) (Sacrificed day 4)	2	only 2 of 12 animals survived this procedure	16– 43	
15. Pancreatectomized (day 1) and on day 2, 1000 u. insulin/kg. i.p. (sacr. after 60 min.)	3	243	190–320	

rise to 156 mg.%. Glucagon, the so-called hyperglycemic-glycogenolytic factor of mammals, when administered to lizards produced a relatively high hyperglycemia of 235 mg.%. Conditions, then, which usually produce hyperglycemia in mammals (glucose, adrenaline, alloxanization, hydrocortisone, and glucagon) likewise stimulate a hyperglycemia in lizards.

The response to insulin is different in lizards than it is in mammals and amphibians and more similar to the situation in birds (1). Lizards with-

stand relatively tremendous doses (10,000 U/kg. or more) of commercial mammalian insulin preparations and after a brief initial period (10–20 minutes) of hypoglycemia may become mildly hyperglycemic. After 20–21 hours, there may be a mild hypoglycemia. After 4 days the blood sugar returns to normal.

Coulson and Hernandez (5) state that alligators become hyperglycemic after insulin, and like our lizards show a delayed hypoglycemia occurring about 20 hours after the initial injection. The ability of the lizard to recover normal blood sugar levels seems to be somewhat greater than the alligator. We agree with Coulson and Hernandez that the hyperglycemic response is probably an indication of sensitivity to the hyperglycemic factor contained in certain American commercial insulin preparations (35). Our experiments have been limited to the use of Lilly's crystalline zinc insulin and we have not yet tried known hyperglycemic factor-free insulin preparations.

If the alpha cell of the pancreas was protecting the lizard against the effects of insulin or was largely responsible for maintaining an adequate blood sugar level, then destruction of these elements by cobaltous chloride or Synthalin A should render the animal hypoglycemic. Cobaltous chloride effected a mild hyperglycemia but no marked changes in the islet cells. Synthalin A, alone, or in combination with cobaltous chloride, produced a severe fall in blood sugar, but only mild toxic effects on the alpha cells. After Synthalin A, all animals exhibited symptoms suggestive of hypoglycemia (muscular tremor, spasticity, and incoordination) and several expired of acute intoxication. Although we did not study the effects on the liver and kidneys, we agree with the opinions of Davis (6) and Bodo and Marks (7) that the toxic and hypoglycemic effects of Synthalin A are probably due to factors other than a disruptive functional obliteration of the pancreatic alpha cells.

In hypophysectomized animals the average effect was a mild hypoglycemia, although some individuals had a very low blood sugar. We did not section the heads after operation, believing that gross inspection with a dissecting microscope was adequate to determine the completeness of pituitary removal. However, it is possible that remnants of microscopic bits of pituitary gland were responsible for the failure to achieve a greater degree of hypoglycemia in some animals.

Instead of becoming hyperglycemic as do most mammals, amphibians, and even turtles (8), completely pancreatectomized lizards become severely hypoglycemic. A similar situation has been reported to occur in the completely pancreatectomized duck (2). Although it has been suggested that the very radical procedure required for total pancreatectomy of a lizard may impair the portal circulation and thus contribute to a condition of hypoglycemia, histological examination of the livers of pancreatectomized animals did not show cellular damage. Tentatively, we believe that the blood sugar economy of the lizard (and probably also of the bird) is

highly dependent upon a factor which maintains the blood glucose level (glucagon) as well as upon a factor enabling it to use its carbohydrate (insulin). Therefore, we agree with Mialhe (2) that in these vertebrate types (certain reptiles and birds), pancreatectomy expresses itself primarily as a glucagon lack, while in mammals and amphibians, pancreatectomy expresses itself primarily as an insulin deficiency.

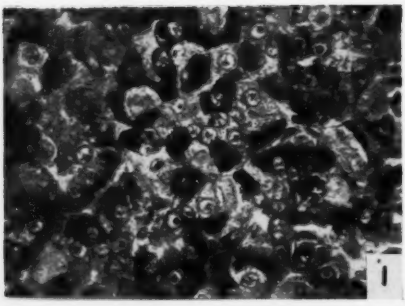
If the alpha cell is necessary to protect the lizard against the hypoglycemic effects of insulin, then the pancreatectomized animal should become hypoglycemic after insulin administration. The result of this procedure, however, was the evocation of a greater hyperglycemia (190–320 mg.%) than occurs after either insulin or glucagon is given to a normal animal. Until we are able to study the effects of glucagon free insulin and glucagon in lizards under many different circumstances, we can only tentatively conclude that the pancreatectomized lizard, an animal without beta cell protection, is even more responsive to glucagon than is a normal animal, and that glucagon is more effective in raising the blood sugar than insulin is in lowering it. Thus, it becomes more and more apparent that lizard insulin and glucagon should be prepared and studied in comparison to the effects of mammalian insulins and glucagons.

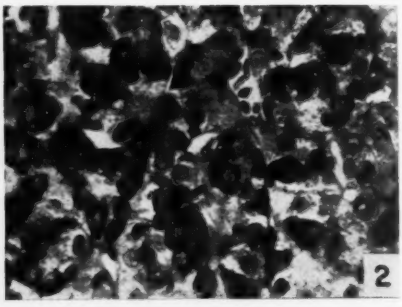
Histological observations

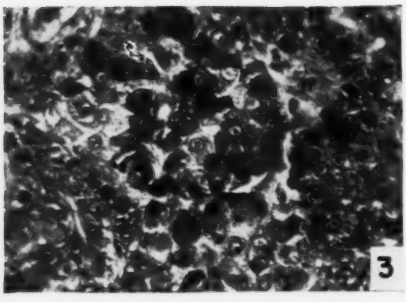
More extensive studies on the islet cells of lizards have reconfirmed our opinion that there are but two basic types of islet cells, namely, the alpha and beta cells (Fig. 1). Under normal circumstances all stages of granulation and degranulation of these cells may be observed. In regard to the argyrophilia of pancreatic cells it has been observed that in frozen sections of formalin fixed tissues stained with the Bielschowski-Gros method, a few alpha cells have very darkly staining granules. In Bouin or formalin fixed paraffine sections stained with a large variety of silver stains, the argyrophilia of the alpha cell is much less intense. However, it seems certain that only a small percentage of the alpha cells possess argyrophilic granules.

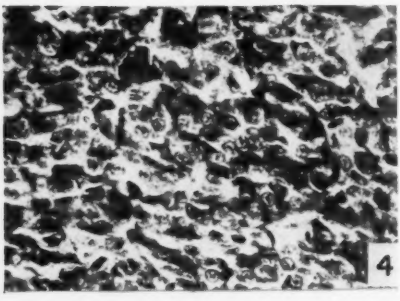
In an earlier paper (1) we reported that glucose administration produced beta cell degranulation, and starvation resulted in alpha cell degranulation, Insulin treatment was interpreted to elicit alpha cell storage in most cases. Further observations after chronic insulin administration has shown, however, that more frequently the alpha cells are degranulated and that many alpha cell nuclei are hyperchromatic (Fig. 2). We interpret the latter as evidence of a strong stimulus having been exerted on the alpha cell.

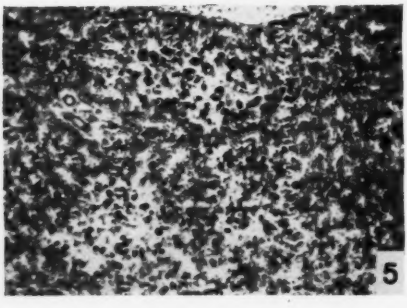
Adrenaline, glucagon, and hydrocortisone, which are hyperglycemia producing agents, lead to moderate beta cell degranulation. The degree of beta cell degranulation is greatest with hydrocortisone (Fig. 4). Not only are the beta cells very much degranulated, but also the staining capacity of the beta granules is much impaired. At the same time, the alpha cells show a greater degree of granular storage than in any other condition. As re-

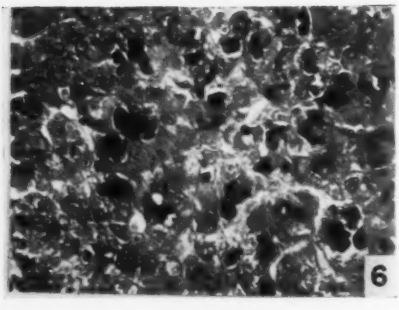












EXPLANATION OF FIGURES

The tissues in all figures were Bouin fixed and stained with Gomori's chromalum hematoxylin and phloxine. The dark cells are the beta cells and the gray cells are alpha cells. Figures 1, 2, 3, 4, and 6 are ×400 and figure 5 is ×200.

FIG. 1. A normal aimal showing alpha (gray) and beta (black) cells in various stages of granular storage and degranulation.

FIG. 2. An animal treated with 2000 U./kg. of insulin daily for 4 days and sacrificed 60 minutes after the last injection. The beta cells are well filled with granules. The alpha cells are degranulated and many of them show hyperchromatic nuclei.

FIG. 3. An animal treated with Synthalin A for 4 days. Most alpha cells are highly degranulated and some show signs of dissolution. Some of the beta cells are also degranulated.

ported earlier (1) alloxanization produces beta cell destruction (Figs. 5 and 6) and concomitant hyperglycemia. The alpha cells are in a storage state in n the alloxanized lizard.

Synthalin A when given in sub-toxic dosages leads to a considerable degree of alpha cell degranulation, and a few cells show a homogeneous or "washed" appearing cytoplasmic mass instead of discrete granules (Fig. 3). Only in very few areas was actual cellular disruption observed. If the severe hypoglycemia which accompanies synthalization were due to alpha cell damage, it is not apparent from morphological examination. As we stated above, we believe other factors than alpha cell intoxication are responsible for the hypoglycemia.

After hypophysectomy there is a mild increase in both alpha and beta cell degranulation. Probably longer periods subsequent to hypophysectomy are necessary to produce more definite cellular alterations.

DISCUSSION

The amount of biochemical data for reptiles is relatively scarce as compared to that available for other vertebrates. Our average normal fasting blood sugar for *Eumeces obsoletus* (112 mg.%) is similar to the values reported in other reptiles. For the lizard, *Anolis carolinensis*, Dessauer (9) reports a minimum seasonal average of 110 mg.% and a maximum of 197 mg.%. In snakes, Prado (10) gives an average of 54–58 mg.% for *Bothrops*, and Rhaney (11) 91 ±4 mg.% for *Lampropeltis*. In the turtle, *Phrynops*, Foglia *et al.* (8) report a level of 76 mg.%, and in the alligator, Coulson and Hernandez (5) indicate a normal level of 50–100 mg.%.

We have previously discussed the normal histology of reptilian islets (1). In regards to the argyrophilia of the alpha cells, our findings are similar to those of Creutzfeldt (12) in the dog where argyrophilia is best demonstrated when frozen rather than paraffine sections are treated with silver methods. As has been found in mammals (13, 14), in birds (15, 16), and by us in lizards, a great deal of species variability exists in the degree of argyrophilia of alpha cell granules. One must agree with the contention of Burkl (14) that, under normal circumstances, not all alpha cell granules demonstrate argyrophilia, and that sometimes even beta cell granules may exhibit this characteristic. Thus, argyrophilia is not necessarily a characteristic of alpha cell granules.

FIG. 4. An animal treated with 20 mg./kg. of hydrocortisone every other day ×3 and sacrificed on day 7. The darker cells in this case are alpha cells which are both compressed by the increased volume of beta cells and are well filled with granules. The cells with the empty appearing cytoplasmic spaces are the highly stimulated and degranulated beta cells.

FIG. 5. An alloxan treated animal showing marked destruction of the beta cells (black masses).

FIG. 6. A higher power view of an alloxan treated animal showing disintegrating beta cells. Some of the alpha cells also evidence signs of toxicity.

As has been reported for members of all vertebrate classes, adrenaline provokes hyperglycemia in lizards and other reptiles [in snakes (10), and alligators (5)]. Coincident with the hyperglycemia produced by glucose feeding or adrenaline, one finds degranulation of beta cells [see review by Bargmann (17)].

In mammals, relatively large doses of hydrocortisone produce hyperglycemia with coincident beta cell degranulation. Interestingly in *Eumeces* the stimulation of the beta cells is so intense that not only is there extreme degranulation, but also the staining capacity of the granules is greatly impaired. Perhaps with longer stimulation actual cellular atrophy might ensue as occurs in some humans undergoing prolonged hydrocortisone therapy.

As one descends the ladder of vertebrate orders, the degree of resistance of the pancreatic beta cell to alloxan apparently increases. Generally, mammals are relatively sensitive. In a variety of birds (owls, pigeons, ducks and chickens), Scott *et al.* (18) were unable to damage the beta cells, while Goldner and Gomori (19) on the other hand, were able to produce a hyperglycemia in the pigeon. We have shown in the lizard that after multiple doses and a 10–14 day wait, both hyperglycemia and beta cell damage can be produced. Likewise in the turtle, Garcia-Ramos (20) has evoked a hyperglycemia. In frogs, Seiden (21) showed a hyperglycemia together with beta cell changes. In the salamander, Wurster (22) was unable to demonstrate either hyperglycemia or destructive beta cell changes. In fishes, both Schatzle (23) and Pallot (24) have reported alloxan resistance.

Mammals in general are relatively insulin sensitive, while both birds and reptiles have been shown to be insulin resistant (1). Alligators, although relatively insulin resistant (5) are apparently less so than lizards and snakes (10).

We, in agreement with Coulson and Hernandez (5), feel that much of the "resistance" exhibited by reptiles to insulin may be due to the response of the reptile to the hyperglycemic-glycogenolytic factor contained in most mammalian insulin preparations.

Our experiments indicate that the lizard is very sensitive to glucagon, responding rapidly with an average hyperglycemia of 235 mg.%. Studies by Hazelwood and Lorenz (25), Mialhe (26), and Snedecor *et al.* (27) have shown that birds are also very glucagon sensitive. Vuylsteke and de Duve (28) have demonstrated that the bird pancreas contains six times as much glucagon as the mammalian pancreas.

The great sensitivity of lizards to mammalian glucagon suggests that the so-called "insulin resistance" of reptiles may be due to the marked and long enduring hyperglycemic effect of glucagon in these animals. We believe also that the relatively high hyperglycemia produced by insulin after pancreatectomy can be explained by an increased sensitivity of the lizard to the hyperglycemic factor of mammalian insulin when the animal has no opposing beta cell hormone of its own. In sharp contrast to the glucagon

sensitivity of reptiles and birds is the almost total insensitivity found in salamanders (29). Correlatively, the pancreases of birds and reptiles contain an abundant amount of alpha cell tissue while those of salamanders are lacking in these elements (29).

Of particular interest is the apparently great divergence in metabolic behavior occurring between some of the various orders of certain vertebrate classes. As an example, the factors concerned in blood sugar regulation in the toad (30) are much different from those in the salamander (29). Similarly, pancreatectomy in the turtle (8) produces a hyperglycemia but hypoglycemia in the lizard (see below).

The effects of cobaltous chloride and Synthalin A in lizards are similar to those observed in most mammals (31, 32, 33) and in birds (34). Because of the relatively small amount of observable alpha cell damage, we believe the severe hypoglycemia and shock produced by Synthalin A is probably due to extrapancreatic effects (see above under Observations). Along with most other observers (31) we feel the results produced by both cobaltous chloride and Synthalin A are far from conclusive in helping assess the role of the alpha cell in carbohydrate metabolism.

The fall in blood sugar after hypophysectomy is in keeping with the situation in all other vertebrate animals. Had a longer time elapsed after hypophysectomy and examination of the pancreatic islet cells, more definite changes might have been observed.

However, pancreatectomy elicits a response entirely at variance with the biochemical situation in any other vertebrate except the duck (2). Both the duck (2) and the lizard become hypoglycemic following complete pancreatectomy. Agreeing with Mialhe (2), we attribute the hypoglycemia following pancreatectomy to the lack of a blood sugar maintaining and elevating factor (glucagon) in types of organisms which are highly dependent upon it. We also agree with Mialhe (2) that, even if small remnants of pancreas remain, a marked hypoglycemia will not occur.

The seeming paradox of a marked hyperglycemia occurring after insulin administration to pancreatectomized animals can possibly be explained by the presence of glucagon in the insulin preparations which we used. Furthermore, an animal already sensitive to this substance may be especially sensitive in the absence of any beta cell insulin of its own type. One might suggest also that the lizard is much more sensitive to the mammalian hyperglycemic-glycogenolytic factor than it is to mammalian insulin. A complete analysis of this problem must await the isolation and study of lizard insulin and glucagon.

The contrast of the hypoglycemia following pancreatectomy in lizards to the hyperglycemia following pancreatectomy in turtles (8) is another example of important metabolic differences existing between the orders of certain vertebrate classes. In birds (at least in ducks) and lizards, pancreatectomy expresses itself as a glucagon lack (with resulting hypoglycemia), while in mammals, in turtles among the reptiles, in amphibians,

and in fishes, it expresses itself as an insulin deficiency (with resulting hyperglycemia).

The marked sensitivity of the lizard to glucagon, together with the phenomenon of hypoglycemia following pancreatectomy would indicate a pancreatic source of the hyperglycemia producing material and the importance of its role in lacertian carbohydrate metabolism. Final proof of the relation of the alpha cell to the hyperglycemic factor is lacking. However, indirect evidence such as the stimulation of the alpha cell by chronic insulin administration and by starvation implicates it as a likely source of the hyperglycemic factor.

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EFFECTS OF A BACTERIAL POLYSACCHARIDE (PIROMEN®) ON THE PITUITARY-ADRENAL AXIS: CORTISONE BLOCKADE OF PIROMEN-INDUCED RELEASE OF ACTH

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ABSTRACT

The bacterial polysaccharide, Piromen, stimulates the pituitary to release ACTH. The intensity of the response varies with the dose of the drug employed. Certain aspects of the increased adrenal activity following Piromen are somewhat atypical, e.g., failure of the thymus to involute in animals, and positive nitrogen balance in humans. An attempt was made to determine whether the ACTH-releasing effects of this drug could be blocked by pre-existing levels of steroid. Male, 100 gm. rats were pre-treated with cortisone, desoxycorticosterone, followed by a triggering dose of Piromen known to cause ACTH release. When adequate time was given to effect complete absorption of cortisone (pre-injection) the response to Piromen was completely blocked. Desoxycorticosterone had no effect. It is concluded that the pituitary-stimulating effects of Piromen are largely governed by pre-existing levels of circulating steroids especially of the glucocorticoid variety.

INTRODUCTION

PREVIOUS reports (1, 2) have shown that the bacterial polysaccharide complex, Piromen®[2] stimulates the pituitary gland to release ACTH. Certain facets of this pituitary-adrenal activation are quite different from those found in the conventional response to ACTH; experimentally, these are the degree, duration, and character of the response shown by ascorbic acid, cholesterol and histologically demonstrable lipids in the adrenal. Hypophysectomy completely abolishes these responses (2). Adrenocorticotropic effects which follow the prolonged administration of Piromen resemble, in the intact animal, those seen when ACTH is given, but these responses are not associated with the usual catabolic sequelae. Growth continues, but involutionary changes in thymus and spleen are absent. In

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[2] Piromen is a sterile, non-protein and non-anaphylactogenic bacterial polysaccharide complex. It is prepared as a stable suspension in 1/6 *M* sodium r-lactate containing 1:10,000 parts of thimerosal. Piromen is a product of Travenol Laboratories, Inc., a subsidiary of Baxter Laboratories, Inc., Morton Grove, Illinois.

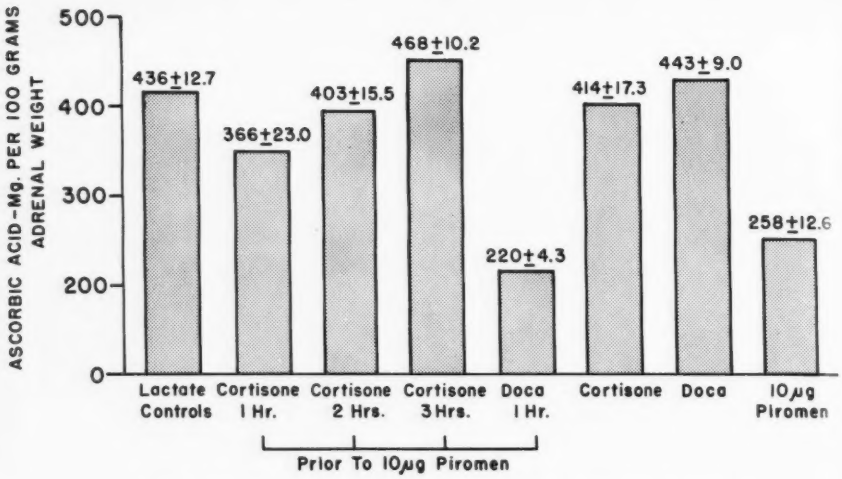

FIG. 1. Cortisone blockade of Piromen-induced release of ACTH.

these observations, as in those with human subjects (3) the metabolic and anatomic responses to the bacterial polysaccharide can be best explained by the simultaneous release of both ACTH and growth hormone.

Sayers has shown (4) that circulating adrenal steroids determine the release of endogenous ACTH and that the steroids vary in their potency to suppress endogenous ACTH release. Because of the different metabolic sequelae which follow Piromen we wished to determine whether the endogenous release of ACTH initiated by Piromen would be blocked in part or completely, or be unaffected by pre-treatment with cortisone.

MATERIALS AND METHODS

Sprague-Dawley male rats were used. They were housed in air-conditioned quarters and fed Purina laboratory chow *ad libitum*.

RESULTS

The usual response to Piromen was blocked by pre-treatment with cortisone (Fig. 1). Inhibition of endogenous release of ACTH by Piromen was most effective as the time interval between pre-treatment and the injection of Piromen was lengthened. Perhaps the increased time interval permitted better absorption of cortisone. Complete inhibition occurred when cortisone was given three hours before Piromen was injected. Under the conditions employed, DCA did not block ACTH release. No significant reduction in ascorbic acid levels was found after cortisone or desoxycorticosterone administration. The potency of the Piromen used for inducing endogenous ACTH release was demonstrated because at a dosage of 10 μg. it

induced a fall in ascorbic acid from the normal level of 440 mg.% to 250 mg.%. This is the expected response (1).

DISCUSSION

Our results indicate that sensitivity of the organism to Piromen, if ACTH release be considered, is dependent upon the blood titre of circulating steroids, particularly glucocorticoids. It was observed that when the maximum time for cortisone absorption was allowed (3 hours) the ascorbic acid levels in the adrenal glands were elevated. Here the pituitary gland may have been blocked and ACTH secretion completely inhibited. By contrast pre-treatment with DCA 1 hour before giving Piromen was followed by a maximal decline in ascorbic acid levels showing no blockade whatsoever; this would suggest that DCA and cortisone have opposing effects on the pituitary-ACTH release mechanism (4–6).

Animals weighing 100 ± 10.0 gm. were placed in each of 8 groups. Group I, consisting of 30 rats, was reserved for control purposes. Each rat was given 0.1 cc. of M/6 sodium r-lactate, the Piromen diluent, intraperitoneally (i.p.) for each 100 gm. of body weight (b.w.). Animals in Group II (20 rats) received 5 mg. of cortisone acetate subcutaneously (s.c.) for each 100 gm. b.w.; 1 hour later 10 μg. of Piromen/100 gm. b.w. was administered i.p. Animals in Group III (12 rats) were given the same amount of cortisone 2 hours preceding the injection of Piromen; those in Group IV (12 rats) were given equivalent amounts 3 hours before Piromen.

Desoxycorticosterone acetate (DCA) is least effective of the available steroids for blocking endogenous ACTH release (4–6). Hypothetically, it should also interfere least with the response to Piromen. We tested the validity of this assumption by giving 5 mg. of DCA for each 100 gm. of body weight to animals in Group V (12 rats) and followed this with 10 (μg.) of Piromen/100 gm. b.w. 1 hour later. Cortisone alone was injected s.c., 5 mg. per 100 gm. b.w., into animals in Group VI (12 rats). DCA (5 mg./100) was given by itself to animals in Group VII (12 rats) and, for the last group (VIII), 10 (μg.) of Piromen per 100 gm. b.w. was injected i.p. as a check on the potency of the preparation.

All animals were sacrificed by decapitation four hours after injection of Piromen, Cortisone or DCA. The adrenals were prepared for the ascorbic acid assay in accordance with methods described in an earlier report (1). The level of adrenal ascorbic acid depletion was used as an index of endogenous ACTH release (6).

Conn (3) and others have found that adrenal stimulation produced in patients varies with the dose of Piromen employed. Doses up to 25 μg. do not elicit detectable increases in steroid levels. Increased amounts of Piromen caused significant eosinopenia and steroid excretion. Our experimental results suggest that endogenous circulating corticoids are able to block the pituitary-stimulating effects of low doses of Piromen.

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THE ANTAGONISM OF SALICYLATE TO DIETHYLSTILBESTROL UPON LIVER GLYCOGEN IN THE RAT[1]

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ABSTRACT

Since salicylate has been reported to antagonize liver glycogenesis after cortisone, an experiment was designed to test the antagonism of salicylate to diethylstilbestrol upon liver glycogen in normal, fasted, female rats.

Liver glycogen levels, elevated by estrogen treatment for two weeks, were found to be lowered by a subsequent injection of sodium salicylate, 5–8 hours before sacrifice. The action of the estrogen in producing liver and adrenal enlargement, moderate depletion of adrenal ascorbic acid, and maximal depletion of adrenal cholesterol was confirmed. Salicylate alone produced only a moderate depletion of adrenal ascorbic acid, while the combined effect of the drugs was seen in a marked depletion of this substance.

Diethylstilbestrol and sodium salicylate were synergistic with regard to the stimulation of activity in the rat adrenal cortex under these conditions (probably by release of ACTH), but their actions on liver glycogen levels were in the opposite directions. The estrogen stimulated glycogen accumulation while salicylate was glycogenolytic; in combination, the substances were antagonistic in this respect. It is concluded that the mechanism is a salicylate-glucocorticosteroid antagonism.

SALICYLATE produces some interesting metabolic effects. It has recently received much attention since the demonstration by Blanchard *et al.* (1) and by others (2, 3, 4) of its ability to stimulate the rat adrenal cortex, apparently through the release of ACTH (1, 3, 4). Paradoxically, Lutwak-Mann (5) has shown that salicylate depletes the rat liver of glycogen, while Feeney, Carlo, and Smith (6) found that the pituitary was not necessary for this effect. M. J. H. Smith (7) found that sodium salicylate was antagonistic to cortisone upon liver glycogen levels in adrenalectomized rats. Edelman, Bogner, and Steele (8) found the antagonism extended to normal rats receiving a glucose load, and Winters and Morrill (9) have shown that fasting rats treated with salicylate fail to deposit liver glycogen after cortisol.

Since many estrogens bring about the accumulation of glycogen in the liver of normal rats (10, 11, 12, 13), presumably by adrenal cortical stimula-

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[1] A preliminary report of these studies has been published (Teague, R. S. and G. O. Barnett, XXth International Physiological Congress, Abstracts, p. 880, 1956).

[2] Lederle Medical Student Fellow, 1955.

tion through ACTH release (11, 14), it seemed of interest to study the effect of salicylate upon these responses to diethylstilbestrol.

METHODS

An experiment was designed to study the acute effect of one injection of salicylate upon the liver glycogen level after it had been elevated by a course of daily estrogen injections for two weeks, and at the same time to observe the changes in the adrenal ascorbic acid and cholesterol concentrations.

Normal, adult, female, Sprague-Dawley rats of the departmental colony, maintained on Purina Laboratory Chow, were used in four groups of six rats each. They were caged separately, injected at staggered intervals, and the data subsequently pooled. A *Stilbestrol + Salicylate Group* received a daily subcutaneous injection of 1 mg. of diethylstilbestrol in 0.5 cc. of cottonseed oil (Lilly) for 14 days. On the morning of the 14th day, the last estrogen injection was given and food was removed from the cage. On the morning of the 15th day, a subcutaneous injection of a suspension of about 100 mg. of sodium salicylate (U.S.P., Merck) in 0.5 cc. of sesame oil was administered. The animals were sacrificed five to eight hours later (29–32 hours after the removal of food and after the last estrogen injection).

A *Salicylate Group* was treated similarly except that daily cottonseed oil injections were substituted for the estrogen. Another lot, a *Stilbestrol Group*, received the estrogen injections but sesame oil was substituted for the salicylate. Finally, an *Oil Control Group* received appropriate oil injections throughout the experiment.

The animals were tame and were handled gently at all times to avoid undue excitement. At the time of sacrifice, they were exsanguinated under Dial-Urethane[3] anesthesia by severing the great abdominal vessels. About one gram of the right lateral lobe of the liver was quickly removed, blotted, weighed, and wet ashed in hot KOH for glycogen determination (15). Then one adrenal was rapidly removed, trimmed, weighed on a microtorsion balance, and immediately homogenized (Potter-Elvehjem) in 4% trichloracetic acid for ascorbic acid analysis (16). The other adrenal was then similarly weighed homogenized in hot alcohol-acetone for total cholesterol determination (17). Finally, the remainder of the liver was removed, blotted, and weighed. With a team of assistants, the time between the injection of the anesthetic and tissue digestion was cut to a minimum.

Statistical Analysis. The data collected in the four groups of animals comprised, for each set of measurements, a two-by-two factorial design, to which was applied an analysis of variance with multiple classification, as described in section 11.8 of Snedecor's text (18). In this manner, the effect of the estrogen and of the salicylate could be assessed independently, and any discrepance or interaction between the two treatments could be weighed.

RESULTS

The data are summarized in Figure 1 and an analysis of the findings is shown in Table 1. As was expected, the liver glycogen concentration, elevated by estrogen, was found to be lowered after subsequent treatment with salicylate. Salicylate alone produced extremely low glycogen levels. The interaction with respect to glycogen shown in Table 1 fell below the level of significance after logarithmic transformation.

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[3] The Dial with Urethane Solution was generously supplied by the Ciba Pharmaceutical Products, Inc.

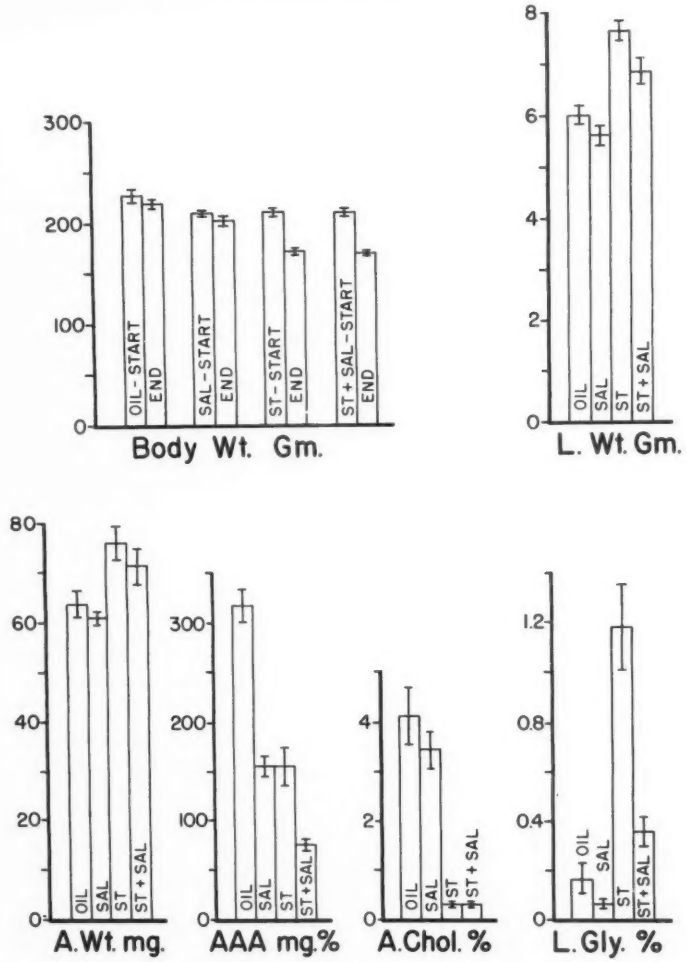

FIG. 1. Bar diagrams of the means ± one standard error of the mean of the data in four groups of six rats. OIL: group receiving oil injections alone. SAL: group receiving oil injections for 14 days and sodium salicylate the 15th day. ST: group recieving diethylstilbestrol daily for 14 days and oil alone the 15th day. ST + SAL: group receiving diethylstilbestrol 14 days and sodium salicylate the 15th day. Fast period 29–32 hours. Body Wt. Gm.: the body weight at the beginning and at the end of the experiment. L. Wt. Gm.: the liver weight in grams. A. Wt. mg.: the adrenal weight in milligrams. AAA mg.%: the adrenal ascorbid acid concentration. A. Chol. %: the adrenal total cholesterol concentration. L. Gly. %: the liver glycogen concentration.

The accompanying adrenal changes are of interest; the weight of the adrenal was increased by the estrogen injections but was not affected by the acute salicylate treatment. Adrenal ascorbic acid was moderately

TABLE 1. ANALYSIS OF VARIANCE AND TESTS OF SIGNIFICANCE. EFFECT OF TREATMENT WITH DIETHYLSTILBESTROL, SALICYLATE, AND THEIR INTERACTION

Parameter \ Source of variation	Mean squares			
	Stilbestrol treatment	Salicylate treatment	Interaction	Error
Adrenal Weight	780.3**	95.4	4.9	53.1
Adrenal Ascorbic Acid Conc.	85,443**	85,443**	10,086**	1201
Adrenal Cholesterol Conc.	72.530**	.620	.584	.782
Liver Weight	12.355**	2.209**	.216	.237
Liver Glycogen Conc. %	2.530**	1.251**	.7708**	.0561
Log Liver Glycogen Conc. (mg.%/10)	4.100**	1.053**	.0582	.0533

** Probability <.01.

depleted by the estrogen and by the salicylate administered separately, but it was extremely depleted by the combination. This indicates a synergistic (additive) action in stimulating the release of ACTH, as measured by the adrenal ascorbic acid. On the other hand, the adrenal cholesterol concentration, while not appreciably lowered by acute treatment with salicylate alone, was maximally depleted by the estrogen; it seems unlikely that the additional salicylate treatment could have lowered the cholesterol concentration further.

The action of diethylstilbestrol in increasing the weight of the liver is also indicated in Figure 1; the similarity in the responses of the liver and the adrenal weights in the four groups is striking. It should be noted that the adrenal and liver weight data are actual values, not adjusted for body weights. The increase in liver weight produced by diethylstilbestrol is emphasized in Table 2, where it will be seen that the livers of the *Stilbestrol Group* are heavier than those of the *Oil Control Group*, even though the starting body weights of the former are the smaller. The adrenal weights are included to show that the increase in the liver weight (in this case) is actually more marked than the adrenal hypertrophy, although the latter effect of estrogen is well known and its action in producing liver enlargement is not.

The small but significant decrease in liver weight attributed to salicylate treatment may partly be explained by the differences in starting body weights. The adrenal weight after salicylate similarly decreases but the change does not approach a significant level.

TABLE 2. MEAN BODY AND ABSOLUTE ORGAN WEIGHTS (±STANDARD ERROR OF THE MEANS) IN RATS RECEIVING DIETHYLSTILBESTROL FOR TWO WEEKS

	Body weight, gm.		Adrenal weight, mg.	Liver weight, gm.
	Start	End		
Oil Control Group	226 ± 7	218 ± 5	63.8 ± 2.9	6.01 ± .18
Stilbestrol Group	209 ± 4	171 ± 3	76.1 ± 3.5	7.63 ± .18

DISCUSSION

A superficial explanation of the endocrine mechanism of these changes (for which there is much evidence) is that the estrogen brings about a chronic increase in ACTH discharge from the anterior pituitary, reflected in the adrenal hypertrophy and depletion of adrenal ascorbic acid and cholesterol. The resulting chronic increase in circulating glucocorticosteroids raises the liver glycogen levels. It may be assumed that salicylate can superimpose an additional acute release of ACTH, as revealed by the further fall in adrenal ascorbic acid, but not by the cholesterol concentration since it is already maximally depleted. We thus have evidence for an additive action of salicylate and diethylstilbestrol in releasing ACTH. The significant interaction in regard to adrenal ascorbic acid (Table 1) may be explained by the fact that this response (to ACTH) is curvilinear at high dose levels.

In spite of the additional adrenal cortical stimulation produced by salicylate after the estrogen, liver glycogen levels are found to fall instead of rising further; since salicylate is known to antagonize the glycogenic action of cortisone, it follows that salicylate similarly antagonized diethylstilbestrol's glycogenic action, indirectly produced by the discharge of glucocorticosteroids.

However, certain observations are not in complete agreement with this, possibly oversimplified, explanation. Smith (19) was unable to demonstrate antagonism of salicylate to cortisone or cortisol on glycogen levels in rat liver slices, but found a slight antagonism to an adrenocortical extract. Ingle (20) has shown that diethylstilbestrol, under certain conditions, has a diabetogenic action in the rat independently of the adrenals, although the presence of adrenal steroids modifies the response. The diabetogenic effect of the estrogen and cortisone are synergistic (21). Yet the estrogen has long ago been reported to increase the amount of extractable insulin in the rat's pancreas (12), and it has been shown to produce islet hyperplasia (22). The extent to which epinephrine, insulin, glucagon, growth hormone, and other pituitary hormones are involved in our experiments can only be surmised.

It should be noted that the adrenal response to diethylstilbestrol (hypertrophy, moderate depletion of ascorbic acid, extreme depletion of cholesterol, and extreme decrease of sudanophilia) is similar to the type III response of Sayers (23), typically seen in continuous, severe stress ending in collapse and death. Our animals, however, seemed to be in good condition, and it is difficult to accept estrogen in the role of a severe stressing agent (24). Our findings with respect to the effect of diethylstilbestrol upon adrenal ascorbic acid and cholesterol are in general agreement with those of others, but some workers feel that both estrogen (25) and severe stress (26) produce adrenal changes, reflected in the decreased sudanophilia, which cannot be ascribed solely to ACTH. On the other hand, there is much evidence that the

stimulating effect of estrogen is not directly upon the adrenal cortex, and, in fact, McKerns (27) has recently shown that high concentrations of estrogens directly inhibit adrenal cortical secretion *in vitro*. The response of the adrenal peculiar to estrogen may eventually be explained by the suggestion that there may be more than one ACTH secreted by the pituitary (28).

Regarding the adrenal response to salicylate, in spite of a great deal of evidence that the substance acts through the liberation of ACTH, it should be noted that some claim that the drug may reduce adrenal ascorbic acid in the hypophysectomized animal (29). The cholesterol concentration in the group receiving salicylate alone might have been lower if more time had been allowed to elapse before the rats were killed (23).

At present, the best explanation of the salicylate-estrogen antagonism on liver glycogen would seem to be fundamentally a salicylate-glucocorticosteroid antagonism. Salicylate probably has a more complex effect on carbohydrate metabolism in the liver than a simple or toxic glycogenolysis, since it affects carbohydrate metabolism in the periphery also, and it can decrease diabetic glycosuria in the absence of the adrenals (30, 31). The drug has recently been shown to uncouple phosphorylation (32, 33), a mechanism which may ultimately provide an explanation for these antagonisms on liver glycogen levels.

We have conducted exploratory experiments in which salicylate was administered over a longer period of time, followed by the estrogen. However, 24 hours after salicylate injections were begun, the liver glycogen tended to rise, suggesting that glycogenesis was being stimulated secondarily; this approach was consequently abandoned.

The action of the estrogen in producing a moderate hypertrophy of the liver has been emphasized here because this point has been neglected in the endocrine literature. It has occasionally been noted previously (12, 24), but little is known concerning its significance. The hypertrophy is accompanied by certain histological changes (13), and it has been claimed that the hypertrophy is prevented by hypophysectomy (34). Our experiments suggest that the adrenal and liver hypertrophy are related and further studies of this response are being made in this laboratory.

Acknowledgment

We wish to express our appreciation to Mrs. Louise W. Lokey and Mrs. Bobby F. Teague for assistance in the conduct of these investigations.

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THE INFLUENCE OF DIETARY SODIUM, POTASSIUM AND DESICCATED THYROID UPON CESIUM-134 METABOLISM IN THE RAT[1]

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ABSTRACT

The effect of desiccated thyroid in simplified (casein-corn starch) rat diets deficient in sodium and/or potassium on the metabolism of cesium-134 was studied using a total of 64 rats. Each rat was fed its respective diet for 7 days before receiving subcutaneously 5 μc. of cesium-134 chloride. A 72-hour balance trial was made.

A weight loss was noted whenever desiccated thyroid was fed or dietary potassium was absent from the diet. Dietary potassium tended to increase excretion of cesium-134 and reduce the cesium-134 content of the tissues. Desiccated thyroid increased excretion of cesium-134 on all diets. In general, the cesium-134 content of the muscle tissue was increased and that of the liver tissue decreased when desiccated thyroid was added to the diet.

BYROM (1) reported that, in myxedema, the diuresis subsequent to thyroid administration is accompanied chiefly by a loss of sodium salt, whereas chiefly potassium salts are eliminated following diuresis brought about in normal subjects by dosage with thyroid. Hyperthyroidism is characterized by an increase in all body functions, a generalized muscular weakness and a loss of body weight. Whenever thyrotoxicosis results in tissue losses, as shown by negative balances of cell nitrogen, potassium must inevitably be released as well (2).

MacLeod and Snell (3) reported that cesium reduced the requirement of potassium in nutrition of lactic acid bacteria. Mraz *et al.* (4, 5) observed an increase in excretion of cesium-134 by the rat when dietary potassium was increased. Hood and Comar (6) reported a relatively large accumulation of cesium in the muscle tissue after dosing with cesium-137.

Cesium is a biologically active fission product of uranium that behaves similarly to potassium and is also considered to be bound intracellularly. It, therefore, appeared desirable to gain information on factors controlling

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the biological behavior of cesium in sodium and/or potassium deficient diets, with or without desiccated thyroid.

MATERIALS AND METHODS

The basal diet (deficient in sodium and potassium) employed was the simplified rat diet used by Mraz and Patrick (7) minus the potassium chloride and with the sodium chloride in the salt mixture replaced by calcium chloride. Sodium chloride, potassium chloride and desiccated thyroid were added to the basal diet at the expense of the corn starch. Three diets were formulated from the basal diet, one containing 0.2% sodium, the second 0.3% potassium and the third 0.2% sodium and 0.3% potassium. The sodium and potassium contents of these diets as determined with a flame photometer are shown in Table 1.

TABLE 1. SODIUM AND POTASSIUM CONTENT OF DIETS AS DETERMINED WITH A FLAME PHOTOMETER

Diet	Na(mg./100 gm.)	K (mg./100 gm.)
Basal (Na and K deficient)	13	14
Basal+0.2% Na	200	16
Basal+0.3% K	17	317
Basal+0.2% Na+0.3% K	240	360

Eight albino rats (Sprague-Dawley strain) were used per diet in each experiment. Four rats of each diet were fed 0.8% desiccated thyroid (0.2% I) in the diet, while the other four were not. Each rat was fed its respective diet *ad libitum* for 7 days before receiving subcutaneously a tracer dose of 5 microcuries (μc.) of cesium-134 chloride and being placed in metabolism units as described by Hansard and Comar (8).

Feces and urine samples were collected daily after initiation of the balance trial. Excreta and tissues were digested with nitric acid and aliquots counted in a scintillation well-type counter. Blood samples were centrifuged and an aliquot of both plasma and red blood cells removed for a determination of cesium-134 content.

In the interest of ease of recording and reading, all data have been reported utilizing a maximum of three significant figures and are not indicative of the sensitivity of the experiments. The 5% level of confidence was the criterion for reporting significance between means.

RESULTS AND DISCUSSION

The data shown in Table 2 were obtained from relatively uniform rats weighing approximately 190 gm. at the time they were started on the diets. The data shown in Table 3 were obtained from relatively uniform rats weighing approximately 110 gm. at the start of feeding of the diets. It may be noted from the weight gains in Tables 2 and 3 that each time desiccated thyroid was fed, a weight depression was experienced by rats receiving it. The addition of potassium to the diet produced weight gains in rats not receiving desiccated thyroid and reduced the weight losses in those receiving it.

The inclusion of desiccated thyroid in the basal diet, Table 2, significantly increased the cesium-134 content of excreta and muscle and decreased its content in the liver and red blood cells. Desiccated thyroid in the 0.2% sodium diet significantly increased excretion of cesium-134 and

TABLE 2. CESIUM-134 CONTENT OF TISSUE AND EXCRETA OF RATS ON DIET WITH AND WITHOUT DESICCATED THYROID, SODIUM AND POTASSIUM

Treatment	Wt. gain (g.)	Tissue* (%/g.)		Blood* (%/ml.)**	Total excretion (%)
		Muscle	Liver	R. B. C.	
Basal	−17 ±4	0.81 ±0.06	0.48 ±0.05	0.17 ±0.03	5.6 ±0.9
Basal+Thyroid	−28 ±4	1.06 ±0.10	0.32 ±0.03	0.10 ±0.01	13.5 ±2.3
0.2% Na	− 8 ±6	0.94 ±0.16	0.46 ±0.04	0.16 ±0.00	5.7 ±1.0
0.2% Na+Thyroid	−24 ±6	1.08 ±0.08	0.27 ±0.03	0.08 ±0.00	15.2 ±0.3
0.3% K	+ 8 ±4	0.66 ±0.18	0.31 ±0.05	0.11 ±0.01	25.4 ±0.9
0.3% K+Thyroid	−10 ±4	0.71 ±0.07	0.26 ±0.04	0.08 ±0.00	28.2 ±2.4
0.2% Na+0.3% K	+24 ±3	0.59 ±0.06	0.27 ±0.03	0.11 ±0.00	18.3 ±0.0
0.2% Na+0.3% K +Thyroid	− 2 ±2	0.75 ±0.10	0.20 ±0.02	0.07 ±0.00	31.2 ±3.4

* Expressed as mean per cent ±standard deviation of subcutaneously administered cesium 134.

** No significant differences in plasma values (which varied from a low of 0.017 to a high of 0.026%/ml.) were found among the treatments.

decreased its retention in the liver and red blood cells. The cesium-134 content of the red blood cells of rats fed the 0.3% potassium diet was significantly decreased by the addition of desiccated thyroid while the differences in excreta and liver content of cesium-134 due to thyroid administration approached significance. A significant increase in cesium-134 content of muscle and excreta and reduction in liver was noted in rats fed the potassium and sodium adequate diet when desiccated thyroid was fed.

Desiccated thyroid in the basal diet, Table 3, induced a significant increase in cesium-134 content of muscle and excreta. The cesium-134 content of muscle and excreta was increased and that in the liver decreased when desiccated thyroid was included in the 0.2% sodium diet. In both the 0.3% potassium and sodium and potassium adequate diets, desiccated thyroid significantly increased excretion of cesium-134.

When the data in Tables 2 and 3 were compared, a difference in cesium-134 uptake by the rat tissues was noted which might be attributed to the size of the rat at time of dosing; the rats used to obtain the data in Table 3

TABLE 3. CESIUM-134 CONTENT OF TISSUES AND EXCRETA OF RATS ON DIETS WITH AND WITHOUT DESICCATED THYROID SODIUM AND POTASSIUM

Treatment	Wt. gain (gm.)	Tissues* (%/gm.)		Total excretion* (%)
		Muscle	Liver	
Basal	− 5 ±2	1.46 ±0.15	0.66 ±0.09	4.9 ±0.3
Basal+Thyroid	−18 ±4	1.91 ±0.59	0.60 ±0.11	14.5 ±0.3
0.2% Na	− 3 ±5	1.70 ±0.50	0.63 ±0.02	5.6 ±0.4
0.2% Na+Thyroid	−15 ±1	2.24 ±0.19	0.47 ±0.09	14.0 ±4.1
0.3% K	+ 6 ±4	1.02 ±0.08	0.46 ±0.13	29.6 ±1.8
0.3% K+Thyroid	− 7 ±5	1.31 ±0.07	0.36 ±0.04	37.5 ±1.9
0.2% Na+0.3% K	+26 ±4	1.02 ±0.07	0.38 ±0.03	22.2 ±0.4
0.2% Na+0.3% K+Thyroid	− 4 ±5	1.38 ±0.09	0.31 ±0.04	31.4 ±0.2

* Expressed as mean per cent ±standard deviation of subcutaneously administered cesium-134.

were smaller and had a greater concentration of cesium-134 in the tissues than those used in Table 2. The excretory patterns of cesium-134 of the rats in the two experiments were quite similar with the exception of the rats fed 0.3% potassium with or without desiccated thyroid and those fed 0.2% sodium and 0.3% potassium without desiccated thyroid. The larger rats did not excrete as much cesium-134 on these potassium supplemented diets as did the smaller rats.

It would appear from these data that dietary potassium has a tendency to increase excretion of cesium-134 and reduce the cesium-134 content of the tissues. In general, the cesium content of muscle tissue increased, even though cesium-134 excretion increased and the cesium-134 content of the liver tissue decreased, when desiccated thyroid was added to the diet. This might be explained by the losses in weight experienced by rats fed desiccated thyroid which would result in greater concentrations of the radioisotope in all tissues of the body. However, since liver tends to be a nutrient-storage organ and, therefore, the potassium and cesium-134 are probably in a more dynamic state there, they would tend to be removed and excreted. Dietary potassium, however, would tend to replace that potassium excreted, while only a single dose of cesium-134 was given and, therefore, no appreciable replacement of cesium-134 was possible in this tissue. The increased excretion of cesium-134 on the 0.3% potassium diet when compared to the complete diet might possibly be explained by a saturation of the tissues with potassium in rats fed the 0.3% potassium diet, and, therefore, less uptake by tissues of an intracellular cation like casium-134 would occur.

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PREVENTION OF EXPERIMENTAL HYPERPARATHYROIDISM BY MAGNESIUM AND POTASSIUM SALTS[1]

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ABSTRACT

Young, growing rats were treated with parathyroid extract and NaH_2PO_4. This treatment produced an unusually intense osteoclastic bone absorption. However, in animals which, in addition, received either $MgCl_2$ or KCl, the bone destruction was diminished and, often, osteoblastic new-bone formation occurred. Still, the skeleton never became totally insensitive to the action of the parathyroid extract.

On the other hand, the production by parathyroid hormone plus NaH_2PO_4 of pathologic calcium deposits in the heart and kidney was completely inhibited by the magnesium and potassium salts, at certain dose levels.

It might have been thought that the protective effects of magnesium chloride and potassium chloride are merely due to some local interference with the absorption of the phosphate, which accentuates the action of parathyroid hormone. However, it could be shown that, even when NaH_2PO_4 is given subcutaneously (by the granuloma pouch technique), both $MgCl_2$ and KCl afford protection against parathyroid hormone overdosage.

The protective effect of potassium and magnesium salts appears to be closely linked to the presence of an excess of phosphate. In rats not sensitized with NaH_2PO_4, parathyroid hormone actually increased susceptibility to the specific toxic effects of $MgCl_2$ and KCl, so that many animals died, exhibiting neuromuscular disturbances and brain edema.

It is assumed that NaH_2PO_4 sensitizes, while $MgCl_2$ and KCl desensitize, the body to certain toxic actions of parathyroid hormone, particularly to its ability to produce soft-tissue calcification. On the other hand, the hormone actually decreases the tolerance of the organism to some of the toxic actions (especially the neuromuscular effects) of $MgCl_2$- and KCl-overdosage, unless these particular effects are counteracted by concurrent treatment with NaH_2PO_4.

The experiments furnish additional examples of the selective conditioning by electrolytes for certain hormone effects.

IT WAS recently noted that combined treatment with certain corticoids and NaH_2PO_4 regularly produces large myocardial necroses in rats (1, 2) and that the development of these lesions can be prevented by the concurrent administration of $MgCl_2$ or KCl (3). Combined treatment with

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dihydrotachysterol and NaH_2PO_4 produces an acute, purulent myocarditis, accompanied by the Mönckeberg-type of arteriosclerosis; these changes can likewise be prevented by the simultaneous administration of $MgCl_2$ or KCl (4). Since dihydrotachysterol shares many of the pharmacologic actions of parathyroid hormone, the question arose whether treatment with magnesium or potassium salts could also suppress the manifestations of intoxication produced by this hormone, especially in animals sensitized to it by concurrent treatment with NaH_2PO_4.

MATERIALS AND METHODS

Two hundred-twenty female Wistar rats, with a mean initial body-weight of 50 gm. (range: 38–60 gm.), were subdivided into the 22 equal groups which constitute the three experimental series of this study.

The parathyroid extract used was a special preparation of "Paroidin" (Parke, Davis and Company), and contained 550 I.U./ml.

The salts employed were monosodium phosphate "Reagent" ($NaH_2PO_4 \cdot H_2O$, Fisher Scientific Company), potassium chloride "Reagent" (KCl, Fisher Scientific Company), and magnesium chloride "Reagent" ($MgCl_2 \cdot 6H_2O$, Merck and Co., Inc.). The two last named salts were invariably given by stomach tube, but the route of administration of the NaH_2PO_4 varied, as indicated in the text.

Throughout the experiment all animals were kept exclusively on "Purina fox chow" and tap water.

The first experiment was terminated after four days, the other two after seven days of treatment, by killing all the survivors with chloroform. Immediately after autopsy the heart and kidneys were fixed in neutral formalin, for subsequent staining with hematoxylin-phloxine (to determine the general histologic structure) and with von Kossa's silver nitrate technique (for the histochemical demonstration of calcium). In addition, the distal metaphyses of the femurs, as well as samples of the ribs of each animal, were fixed and simultaneously decalcified in Susa solution, for subsequent staining with hematoxylin-phloxine. All tissues were embedded in paraffin. The intensity of the calcium deposition in the hearts and kidneys, as well as the severity of the osteitis fibrosa (in the femurs and costochondral junctions) were graded in terms of arbitrary scales of 0 to 3. The means of these determinations (with standard errors), as well as the mortality rate, are listed in the Tables.

EXPERIMENTAL

First Experiment

In this series, parathyroid hormone was administered at the daily dose of 0.5 ml., subcutaneously, twice daily, while the NaH_2PO_4, $MgCl_2$ and KCl were all given at the dose of 150 mg., in 2 ml. of water, twice daily, by stomach tube, as indicated in Table 1.

At this high dose level, parathyroid hormone in itself sufficed to produce osteitis fibrosa and marked calcium deposition in the hearts and kidneys, but it did not cause any mortality (group I). Concurrent treatment with NaH_2PO_4 aggravated the osteitis fibrosa only insignificantly, while the cardiac and renal calcification produced by the parathyroid hormone were significantly augmented so that an 80% mortality resulted (Group II). On the other hand, although the rats which received $MgCl_2$ (Group III),

TABLE 1. EFFECT OF ORAL TREATMENT WITH NaH_2PO_4, $MgCl_2$ AND KCl UPON PARATHYROID HORMONE (Ptr-H) ADMINISTRATION (FIRST EXPERIMENT)

Group	Treatment	Severity of lesions (Scale: 0–3)			Mortality (%)
		Bones	Heart	Kidney	
I	Ptr-H	2.0±0.3	1.1±0.25	1.1±0.2	0
II	Ptr-H+NaH_2PO_4	2.4±0.3	1.9±0.28	2.8±0.1	80
III	Ptr-H+NaH_2PO_4+$MgCl_2$	2.1±0.4	0	0	10
IV	Ptr-H+NaH_2PO_4+KCl	2.0±0.25	0	0.3±0.2	50

or KCl (Group IV) in addition to parathyroid hormone and NaH_2PO_4 still exhibited marked osteitis fibrosa, they developed little or no calcium deposition in hearts and kidneys, and their mortality rate was definitely reduced.

The cardiac changes in Groups I and II consisted mainly of calcium deposition in the blood-vessel walls and muscle fibers (Fig. 1). Often, silver-nitrate-tingible calcium granules were clearly visible in apparently still well preserved muscle cells, but in some instances, patches of myocardial nec-

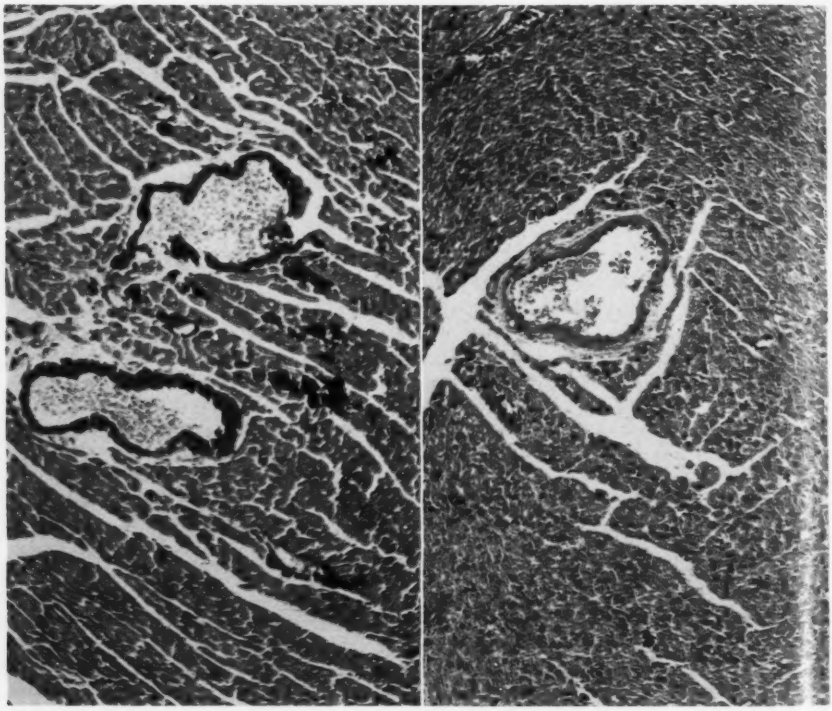

FIG. 1. Intense calcium deposition in the walls of two vessels, as well as in some of the myocardial fibers of a rat treated with parathyroid hormone plus NaH_2PO_4 (left) in comparison with the essentially normal heart of a rat which, in addition to parathyroid hormone plus NaH_2PO_4, received $MgCl_2$ (von Kossa's stain ×120).

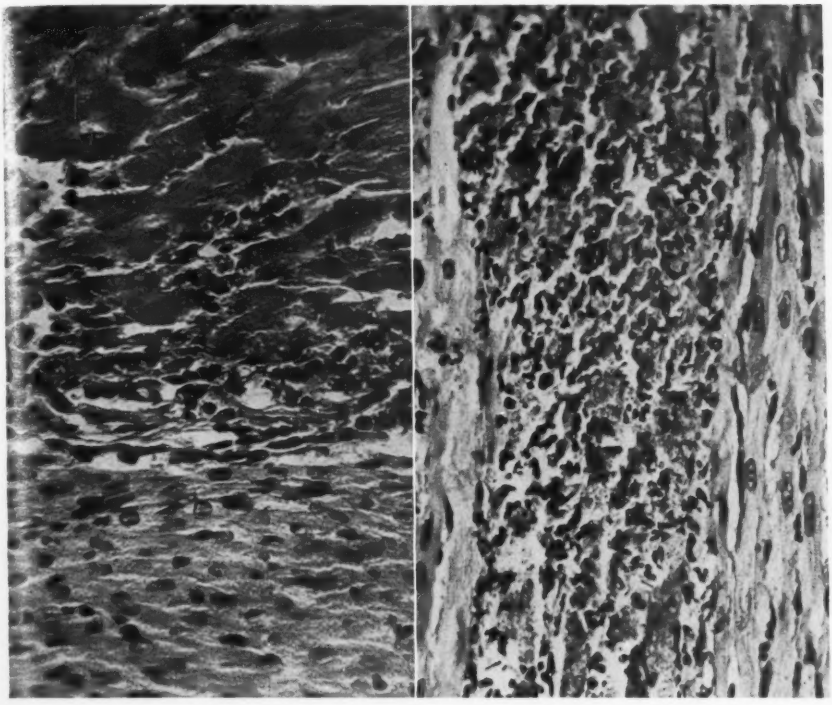

FIG. 2. Cardiac lesions in the heart of a rat treated with parathyroid hormone plus NaH_2PO_4. On the left, necrotic, homogeneous muscle fibres, which form an infarct-like region, sharply demarcated from the normal light-staining cardiac muscle. On the right, somewhat more advanced stage of the same kind of lesion. Here, the necrotic muscle bundle is heavily infiltrated by polymorphonuclear leukocytes. No calcification occurred in either of these foci (von Kossa's stain ×380).

croses and granulomas—similar to those seen in the "infarctoid cardiopathy" (2)—occurred without any sign of calcium deposition (Fig. 2). Apparently, necrosis may or may not precede calcium deposition, depending upon circumstances yet to be elucidated. In Groups III and IV, neither necroses nor calcium depositions were detectable in the myocardium or its vessels.

Nephrocalcinosis was of moderate intensity in Group I and severe in Group II. Most of the calcium deposition was found in the epithelial cells, as well as in the lumina of the tubules at the corticomedullary junction line (Fig. 3). Another, rather constant change in the animals of the first two groups was the development of intense perivascular edema around the larger arteries. On the other hand, nephrocalcinosis was absent in Group III and negligible in Group IV, although the periarterial edema was just as pronounced in these as in the first two groups.

In view of the virtual absence of cardiovascular and renal lesions, the

mortality in Groups III and IV was somewhat unexpected. However, these deaths should be ascribed, not to hyperparathyroidism, but rather to the inherent toxic effects of the comparatively large doses of $MgCl_2$ and KCl, because they occurred without any morphologic evidence of hyperparathyroidism, during the first 60 hours, at a time when all the rats of the first two groups were still alive.

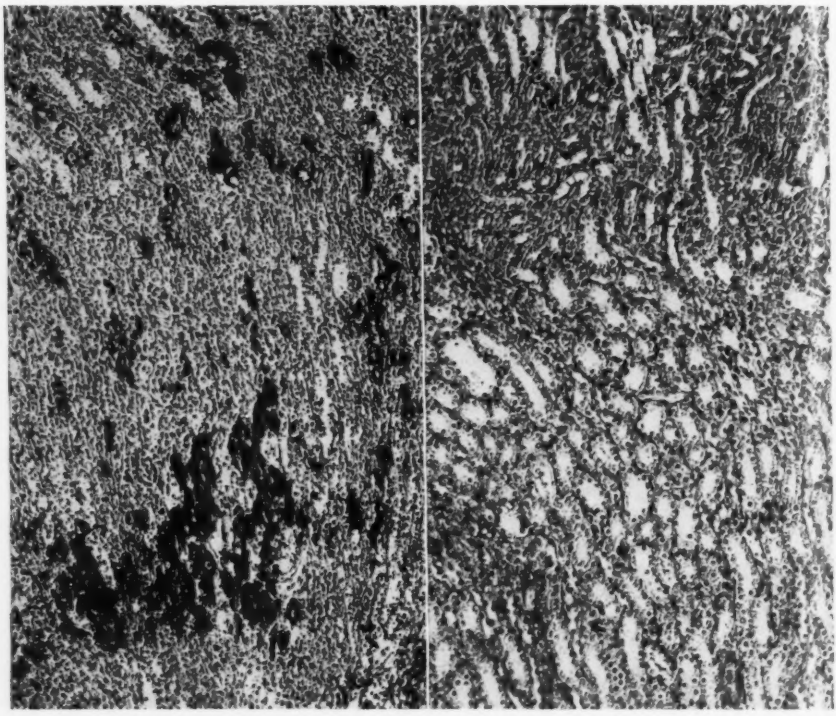

FIG. 3. Intense nephrocalcinosis in a rat treated with parathyroid hormone plus NaH_2PO_4 (left), in comparison with a corresponding region in the kidney of a rat which, in addition to parathyroid hormone and NaH_2PO_4, also received $MgCl_2$ (von Kossa's stain ×100).

Second Experiment

A more extensive experiment was then performed, in which parathyroid hormone was injected at the daily dose of 0.2 ml. twice daily, subcutaneously. NaH_2PO_4 and $MgCl_2$ were again given at the dose of 150 mg., in 2 ml. of water, twice daily, by stomach tube. Because earlier investigators have shown that it is possible to induce some degree of resistance to the toxic effects of potassium salts (5), KCl was administered in gradually increasing doses: 50 mg. during the first, 100 mg. during the second, and 150

mg. during the third and all subsequent days, always in 2 ml. of water, twice daily, by stomach tube. The experiment was terminated on the seventh day.

It is evident from Table 2 that the most intense osteitis fibrosa and soft-tissue calcification again occurred in the animals simultaneously treated with parathyroid hormone and NaH_2PO_4 (Group V). Additional treatment

TABLE 2. EFFECT OF ORAL TREATMENT WITH NaH_2PO_4, $MgCl_2$ AND KCl UPON PARATHYROID HORMONE INTOXICATION (SECOND EXPERIMENT)

Group	Treatment	Severity of lesions (Scale: 0–3)			Mortality (%)
		Bones	Heart	Kidney	
I	Ptr-H	1.0±0.2	0	0.1±0.1	0
II	NaH_2PO_4	0	0	0.1±0.1	0
III	$MgCl_2$	0	0	0	0
IV	KCl	0	0	0	30
V	Ptr-H+NaH_2PO_4	2.0±0.14	0.5±0.1	2.2±0.3	40
VI	Ptr-H+$MgCl_2$	0.2±0.14	0	0	80
VII	Ptr-H+KCl	0.1±0.1	0	0	60
VIII	Ptr-H+NaH_2PO_4+$MgCl_2$	0.7±0.2	0	0.1±0.1	0
IX	Ptr-H+NaH_2PO_4+KCl	0.5±0.25	0	1.2±0.2	20

with either $MgCl_2$ (Group VIII) or KCl (Group IX) exerted a prophylactic effect. However, contrary to out expectations, there was a very high mortality in the animals treated with parathyroid hormone plus $MgCl_2$ (Group VI), or parathyroid hormone plus KCl (Group VIII), without the concurrent administration of NaH_2PO_4, although the latter salt once again aggravated the usual effects (osteitis fibrosa, soft-tissue calcification) of parathyroid hormone intoxication. These observations suggested that NaH_2PO_4 in itself may antagonize the specific toxic effects of $MgCl_2$ and KCL. This supposition has been subsequently confirmed by another experimental series, in which these salts were given alone and in combination to animals that did not receive parathyroid hormone (to be published elsewhere).

In this second experiment (in which parathyroid hormone was given at a much lower dose level than in the first), the osteitis fibrosa was comparatively mild (Group I) and was intensely (and highly significantly) aggravated by concurrent treatment with NaH_2PO_4 (Group V), and ameliorated by $MgCl_2$ (Group VI) and KCl (Group VII). The particularly severe skeletal changes that occur when parathyroid hormone is administered in combination with NaH_2PO_4 (Group V) were likewise diminished both by $MgCl_2$ (Group VIII) and by KCl (Group IX).

In the animals treated with parathyroid hormone alone, or in combination with phosphate, the skeletal lesions were primarily characterized by intense osteoclastic bone absorption, while, in the rats concurrently treated with $MgCl_2$ or KCl, if skeletal changes were detectable at all, they usually

consisted of fibrous tissue proliferation and osteoblastic new-bone formation (Fig. 4). The latter type of response is very reminiscent of the early stages in the "marble-bone type" of proliferative reaction that is normally seen in rats treated with comparatively small doses of parathyroid hormone (6).

It is also noteworthy that the parathyroid hormone, when given without

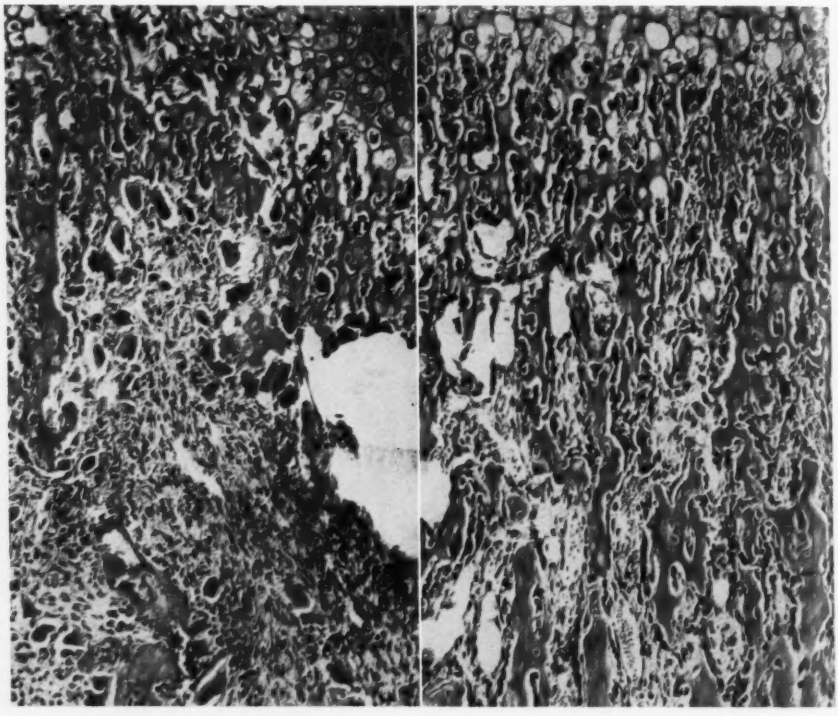

FIG. 4. Intense osteitis fibrosa, with numerous giant cells and cyst formation, in the region just underneath the distal growth-cartilage of the femur of a rat treated with parathyroid hormone plus NaH_2PO_4 (left), in comparison with the corresponding region in the femur of a rat given $MgCl_2$ in addition to parathyroid hormone plus NaH_2PO_4. Note that the magnesium salt did not completely abolish the effect of the parathyroid hormone, but greatly diminished osteoclast formation and bone absorption, although fibrous tissue and osteoblast proliferation are still evident (hematoxylin-phloxine ×100).

phosphate, but in combination with $MgCl_2$ (Group VI) or KCl (Group VII), induced depression, convulsions and pronounced hyperemia as well as edema of the brain. Such changes were not seen in the animals treated with parathyroid hormone (Group I), $MgCl_2$ (Group III), or KCl (Group IV), alone. Apparently, parathyroid hormone actually sensitizes the animal to

certain toxic effects of $MgCl_2$ and KCl, unless the specific toxic actions of these electrolytes are neutralized by the concurrent administration of NaH_2PO_4.

Third Experiment

The possibility still remained that the striking antagonism between phosphate on the one hand and magnesium or potassium salts on the other is merely due to some topical interaction between these electrolytes, which might interfere with the absorption of phosphate from the gastrointestinal tract. This question could be answered by experiments in which $MgCl_2$ or KCl would be administered in the usual manner, *per os*, while the phosphate would be given parenterally. However, it is impossible to administer the required large quantities of phosphate intravenously, subcutaneously or intraperitoneally. Hence, it was decided to introduce them through subcutaneous granuloma pouches. If a subcutaneous air-bubble is prepared under the dorsal skin of the rat and gradually increasing doses of irritants are introduced into the lumen of this cavity, the wall is soon transformed into a highly resistant granuloma pouch which protects the adjacent tissues from necrosis (7).

In view of this, a third experiment was performed in essentially the same manner as the second. However, in order to obtain more pronounced hormone overdosage effects, we administered the parathyroid extract at the dose of 0.2 ml. during the first three days and, after that, at the dose of 0.5 ml. At each level, the hormone was given twice daily, subcutaneously. $MgCl_2$ and KCl were both given at the dose of 50 mg. on the first day, 100 mg. on the second, and 150 mg. on the third and all subsequent days, always in 2 ml. of water, twice daily, by stomach tube. On the other hand, NaH_2PO_4 was administered through subcutaneous granuloma pouches. These were prepared by injecting 20 ml. of air under the shaved skin of the back, under ether anesthesia, and then introducing into the lumen of this cavity 25 mg. of a neutral mixture of NaH_2PO_4 and Na_2HPO_4 in 1 ml. of water. The same dose of this phosphate solution was injected into the pouches, without anesthesia, three more times during the first day. On the second day, 50 mg., on the third and all subsequent days, 75 mg. of the same neutral phosphate solution were injected four times daily into the granuloma pouches. The experiment was terminated on the seventh day (Table 3).

Given at this high dose level, the parathyroid hormone again produced a very intense osteitis fibrosa, about equal in its intensity to that seen in the first experiment; consequently the skeletal changes were less evidently aggravated (by NaH_2PO_4) or inhibited (by $MgCl_2$ or KCl) than in the second experiment, in which the osteitis fibrosa was milder. However, the results were otherwise essentially the same in this as in the previous two experimental series. Hence it may be concluded that the ability of $MgCl_2$ and

KCl to inhibit the intense hyperparathyroidism otherwise obtained by combined administration of parathyroid extract plus NaH_2PO_4 is not due merely to interference with phosphate absorption. Still, it is noteworthy that the magnesium and potassium salts are consistently and markedly effective only in preventing the cardiovascular and renal lesions of parathyroid hormone overdosage when the latter is aggravated by NaH_2PO_4.

TABLE 3. EFFECT OF NaH_2PO_4 (ADMINISTERED THROUGH GRANULOMA POUCH), $MgCl_2$ (ORALLY), AND KCl (ORALLY) UPON PARATHYROID HORMONE INTOXICATION (THIRD EXPERIMENT)

Group	Treatment	Severity of lesions (Scale: 0–3)			Mortality (%)
		Bones	Heart	Kidney	
I	Ptr-H	1.9 ± 0.3	0	0.2 ± 0.1	0
II	NaH_2PO_4	0	0	0.9 ± 0.2	10
III	$MgCl_2$	0	0	0	0
IV	KCl	0	0	0	30
V	Ptr-H + NaH_2PO_4	3.0 ± 0	1.6 ± 0.3	2.6 ± 0.25	50
VI	Ptr-H + $MgCl_2$	2.2 ± 0.3	0	0.3 ± 0.2	80
VII	Ptr-H + KCl	1.3 ± 0.2	0	0	80
VIII	Ptr-H + NaH_2PO_4 + $MgCl_2$	2.0 ± 0.2	0	0	40
IX	Ptr-H + NaH_2PO_4 + KCl	1.5 ± 0.1	0	0	90

DISCUSSION

The striking inhibition of parathyroid hormone intoxication by magnesium and potassium salts that occurred under the experimental conditions described here is somewhat unexpected. However, several earlier observations suggested some relationship between the parathyroids and magnesium metabolism. It has long been known, for example, that the skeleton contains a very large portion of the total body magnesium (8, 9). Furthermore, hypomagnesemic tetany is a definite clinical syndrome, although parathyroidectomy does not appear to produce any constant changes in the blood magnesium level of experimental animals (10–12). Spontaneous hyperparathyroidism, as well as treatment with parathyroid extract, has been claimed to raise the blood magnesium (13, 14), but on this point the literature is quite contradictory (15). The fact that magnesium and potassium act essentially in the same manner in preventing the manifestations of the infarctoid cardiopathy (3, 16), of dihydrotachysterol arteriosclerosis (4), and—as shown by the present experimental series—of the phosphate-aggravated parathyroid hormone overdosage, is especially remarkable because, in several other respects, Mg and K ions are antagonists (17).

It is difficult to understand why, in these experiments, the magnesium and potassium salts markedly antagonized the parathyroid hormone intoxication only when the latter was aggravated by concurrent treatment with NaH_2PO_4. It is possible that when parathyroid hormone is given without NaH_2PO_4, the sensitization by the hormone to the toxic actions of

$MgCl_2$ and KCl (brain edema, convulsions, depression) predominates so much that the beneficial effects (upon the skeleton and soft tissue calcification) of these salts are overcompensated and a high mortality results. Hence, perhaps the milder degrees of hyperparathyroidism—induced in animals not sensitized to parathyroid hormone by concurrent treatment with phosphate—could be effectively combatted only with much smaller doses of magnesium and potassium salts. These points will be further investigated in future experiments, but in any event, the observations reported here show that, under certain experimental conditions, severe hyperparathyroidism can be effectively combatted by the oral administration of $MgCl_2$ or KCl.

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IN VITRO AND *IN VIVO* EFFECTS OF STEROIDS ON GLUCOSE OXIDATION AND RESPIRATION BY NORMAL AND MALIGNANT LYMPHOID TISSUE[1]

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ABSTRACT

A study has been made of the effects of various steroids, added *in vitro*, on the endogenous respiration, oxygen consumption in the presence of added glucose, and oxidation of this glucose to carbon dioxide by whole lymph nodes and by lymphocyte cell suspensions. Rat mesenteric lymph nodes, thymus, and a transplantable lymphosarcoma were used as sources of lymphoid cells. A variety of steroids studied caused inhibition of the metabolism of lymphoid cells, under the experimental conditions used. Correlations are indicated between aspects of steroid structure and inhibitory effects observed. Certain steroids which caused inhibition of metabolism of lymphoid cells when added *in vitro*, were injected into rats 4 hrs. prior to sacrifice of the animals. Lymphoid cells from such steroid-injected rats showed metabolic alterations, when studied *in vitro*, similar to those seen when the steroid was added directly to the cells *in vitro*.

INVOLUTION of lymphoid tissue occurs in a variety of species when blood levels of certain 11-oxygenated adrenal cortical steroids, e.g., hydrocortisone, are elevated as a result of either endogenous release or administration of these steroids (1, 2). The alterations in lymphoid tissue size and histology produced by adrenal steroids have been described in detail (1–3). These effects of adrenal steroids on lymphoid tissue in the absence of the adrenals or hypophysis are mimicked only with three other groups of agents, *viz.*, the nitrogen mustards (4, 5), large doses of deoxycorticosterone and of sex hormones (6–8), and high-energy radiation, e.g., x-rays (9, 10). These observations, together with the demonstration of an *in vitro* effect of adrenal steroids on normal lymphocyte morphology (11–13), have established the lymphocyte as an end cell of adrenal cortical steroid action.

The lymphocytolysis induced by adrenal steroids suggests that these hormones may affect biochemical processes upon which the lymphocyte is dependent for maintenance of its integrity. The inhibition of mitosis seen in lymph nodes shortly following cortisone administration (1–3) is reflected

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in a decreased rate of incorporation of phosphorus into lymphocyte nucleic acids (14–16), as well as a diminished synthesis of nucleic acids (17, 18).

Inasmuch as maintenance of normal lymphocyte structure and function are probably dependent upon energy-producing reactions, studies have been initiated of the *in vitro* and *in vivo* effects of various steroids on carbohydrate oxidation by normal and malignant lymphoid tissue. A number of investigators have examined diverse aspects of the carbohydrate metabolism of normal and malignant lymphoid cells (19–21). The effect of steroids on anaerobic (22) and aerobic (23) glycolysis of lymphoid tissue as well as on the respiration of a variety of tissues *in vitro* (24–26) has been described.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain[2] weighing 140 to 200 gm. were used. The animals were sacrificed by decapitation and the thymus, mesenteric lymph nodes, or transplantable Murphy-Sturm lymphosarcoma[3] were rapidly removed by dissection. The lymphosarcoma was used 2 to 3 weeks following transplantation in the host. Adrenalectomized rats were used 2 to 3 weeks postoperatively. All animals received Rockland rat pellets and water *ad libitum*, except for the adrenalectomized rats which were given 1% NaCl solution as drinking fluid.

In the *in vivo* experiments, a single subcutaneous or intraperitoneal injection of steroid[4], 5 mg./100 gm. of body weight, was made 4 hrs. prior to sacrifice of the animal.

The experiments with mesenteric lymph nodes were conducted with tissue obtained, for the most part, from adrenalectomized rats. The nodes were separated with the aid of forceps into segments approximately 2 mm. in diameter and used in this form. Approximately 50 mg. of nodes were added to a chilled Warburg vessel containing 1.45 ml. of a solution composed of 0.076 *M* NaCl and 9.1×10^{-3} *M* phosphate buffer, pH 7.8. Steroids, when used, were added in highly dispersed aqueous medium[5]; incubations were conducted with oxygen as the gas phase. The center well of the Warburg flask contained filter paper moistened with 0.15 ml. of 10N NaOH. The flasks were then oxygenated and after a preliminary incubation for 10 min. at 37° C in the Warburg bath, uniformly labeled glucose-C^{14} (glucose-U-C^{14})[6] was tipped in from the side-arm to give a final glucose concentration of 3.5×10^{-3} *M*. The manometers were closed and oxygen consumption was

[2] Purchased from Holtzman Co., Madison, Wis.

[3] Initially provided through the kindness of Dr. K. Sugiura of the Sloan-Kettering Institute, and subsequently transplanted in this laboratory.

[4] The steroids used were obtained from the following sources: cortisone, cortisone acetate, hydrocortisone, hydrocortisone-21-acetate, 9-α-fluorohydrocortisone, corticosterone, and cholesterol from Merck, Sharp and Dohme Co.; dihydrocortisone-21-acetate, tetrahydrocortisone-21-acetate, Δ^1-hydrocortisone, Δ^1-cortisone, deoxycorticosterone, deoxycorticosterone acetate, and estradiol from Schering Corp.; 11-deoxycortisone, 11-deoxycortisone-21-acetate, progesterone, dihydrotestosterone, 17α-ethyltestosterone and 19-nortestosterone from Chemical Specialties Co., Inc.; 6-methyl-Δ^1-hydrocortisone and 17α-acetoxyprogesterone from the Upjohn Co.: 17α-hydroxyprogesterone and 17α-ethyl-19-nortestosterone from Searle & Co.; testosterone from Sterol Derivatives, Inc.

[5] Prepared as follows: 4 mg. of steroid were pulverized with a glass rod and suspended in 40 ml. of water with the aid of vigorous mixing.

[6] Purchased from Nuclear Instrument and Chemical Corporation, Chicago, Ill.

measured during 2 to 4 hr. incubation periods with shaking. At the end of this time, 3 ml. of 20% H_2SO_4 were added to each flask to terminate reactions and release all CO_2; the flasks were attached to the manometers again and shaken for an additional 20 min. The alkali paper was removed from the center well, placed in a flask with the washings from the center well, carrier Na_2CO_3 solution added, and the carbonate precipitated with $BaCl_2$. The $BaCO_3$ was filtered, dried and counted for radioactivity in the Nuclear Model 192 Ultrascaler. All counts were corrected to infinite thickness.

In experiments in which thymic or mesenteric lymph node cell suspensions were used, washed cell suspensions were prepared essentially as described by Farber, Kit and Greenberg (27) in their modified Krebs-Ringer phosphate buffer, pH 7.4, using the steps described by Blecher and White (28). Cell counts were made on diluted aliquots of the cell suspensions, which contained approximately 1×10^9 cells per ml.

Because of their greater lability, tumor cell suspensions were prepared by a modification of the procedure used for thymus cells. After mincing the tumor in the cold, it was washed through a #14 mesh Monel metal screen with the Krebs-Ringer buffer. The suspension was then washed successively through #24 mesh and #60 mesh Monel metal screens by gentle agitation; no tissue debris was forced through the screen. This is essentially the procedure described by Kaltenbach and Kaltenbach (29). The cells were centrifuged for 5 min. at 500 R.P.M. and washed again with buffer. After recentrifugation, the cells were suspended in two volumes of buffer.

Incubations with cell suspensions were conducted in Warburg vessels containing 0.5 ml. of cell suspension, 0.25 ml. of the modified Krebs-Ringer phosphate buffer, pH 7.4, and 0.1 ml. of 0.04 *M* glucose-U-C^{14}. In order to maintain tonicity of the medium, steroids when used were first added in acetone solution to the appropriate Warburg flask, the acetone evaporated on a water bath at 60° C under nitrogen, and the cell suspension and buffer added as above. Preliminary incubation and subsequent steps were conducted as described above for mesenteric lymph nodes except that room air was the gas phase.

For each experiment, aliquots of the same lymphoid tissue or lymphoid cell suspension were used with and without steroid and compared with one another under identical experimental conditions.

RESULTS

Influence of Hydrocortisone and Deoxycorticosterone Added in Vitro on Glucose Oxidation by Mesenteric Lymph Nodes

The data obtained from a study of the effect of hydrocortisone and deoxycorticosterone on the aerobic oxidation of glucose-U-C^{14} by mesenteric lymph nodes of adrenalectomized rats are shown in Table 1. The data are expressed as the percentage change in the extent of glucose oxidation and oxygen consumption by the lymph nodes which resulted from addition of steroid. The data indicate that the addition of either hydrocortisone or deoxycorticosterone *in vitro* inhibits significantly the glucose oxidation and oxygen consumption by mesenteric lymph nodes of adrenalectomized rats. The results of this experiment suggest that deoxycorticosterone is somewhat more effective than hydrocortisone in producing this inhibition. This is supported by other data presented later.

Comparative Effects of Hydrocortisone and Deoxycorticosterone Added in Vitro on Glucose Oxidation by Thymic and Lymphosarcoma Cell Suspensions

The metabolism of the lymphosarcoma cells appeared to be more sensitive to the presence of hydrocortisone or deoxycorticosterone than that of

TABLE 1. INHIBITION BY HYDROCORTISONE AND DEOXYCORTICOSTERONE OF GLUCOSE-U-C^{14} OXIDATION AND OXYGEN CONSUMPTION BY MESENTERIC LYMPH NODES OF ADRENALECTOMIZED RATS

Incubation time, hrs.	Steroid	Amount, μg.	Mean % inhibition of glucose oxidation	Mean % inhibition of O_2 consumption
2	Hydrocortisone	20 (1)*	17	23
	Hydrocortisone	40 (6)	20 (11–33)†	20 (9–40)
	Hydrocortisone	80 (1)	27	27
3	Hydrocortisone	20 (1)	2	0
	Hydrocortisone	40 (6)	31 (13–43)	26 (13–37)
	Hydrocortisone	60 (4)	36 (33–40)	25 (12–43)
	Hydrocortisone	80 (5)	34 (25–41)	24 (13–38)
2	Deoxycorticosterone	40 (4)	33 (25–39)	36 (16–53)
3	Deoxycorticosterone	20 (1)	46	22
	Deoxycorticosterone	30 (3)	18 (7–30)	8 (0–24)
	Deoxycorticosterone	40 (1)	80	70

* No. of individual experiments shown in parentheses.
† Values in parentheses under means are ranges of values observed.

normal lymphoid tissue cells (Table 2). Hydrocortisone (180 μg.) produced a two to three times greater inhibition of glucose oxidation, oxygen consumption, and endogenous respiration, of lymphosarcoma cells than of thymic lymphocytes. The inhibitory effects of deoxycorticosterone were significantly greater than those of hydrocortisone for both normal and malignant lymphocytes. The data support those of Table 1 which suggest that the concentration of deoxycorticosterone required to cause a given degree of inhibition of glucose oxidation and oxygen consumption by normal, as well as by malignant lymphoid tissue is lower than that for hydrocortisone. Experiments in which the concentrations of deoxycorticosterone were varied show that 60 μg. of deoxycorticosterone produces the same inhibition as 180 μg. of hydrocortisone. The data also suggest that the lymphosarcoma cells are generally more susceptible to the inhibitory actions of the steroids than are normal thymic lymphocytes.

TABLE 2. INHIBITION BY HYDROCORTISONE AND DEOXYCORTICOSTERONE OF GLUCOSE OXIDATION, OXYGEN CONSUMPTION, AND ENDOGENOUS RESPIRATION OF CELL SUSPENSIONS OF THYMUS AND LYMPHOSARCOMA

Steroid*	Glucose oxidation		Oxygen consumption‡		Endogenous respiration	
	thymus	lymphosarcoma	thymus	lymphosarcoma	thymus	lymphosarcoma
Hydrocortisone	31 (8)†	69 (2)	32 (8)	55 (2)	16 (5)	44 (1)
Deoxycorticosterone	93 (2)	88 (2)	86 (2)	66 (2)	46 (1)	65 (2)

* In each experiment the concentration of steroid was 180 μg.
† Values given are mean per cent inhibition as compared to control without steroid. Values in parentheses indicate number of individual experiments.
‡ In the presence of added glucose.

Effect of Steroid Injection in Vivo on Glucose Oxidation by Lymphocytes in Vitro

The above described inhibitory effects of hydrocortisone and deoxycorticosterone on the oxidation of glucose by thymic and mesenteric lymphocyte cell suspensions were also seen in experiments in which a steroid was injected 4 hrs. prior to removal of the lymphoid tissues for study *in vitro*. These data are shown in Table 3. The uniformity of the data obtained for glucose oxidation by thymic or mesenteric lymphocyte sus-

TABLE 3. EFFECT OF *in vivo* INJECTION OF STEROIDS ON GLUCOSE-U-C^{14} OXIDATION BY THYMIC AND MESENTERIC LYMPH NODE CELLS SUSPENSIONS *in vitro*

Type of animal	Steroid injected	Route of injection	Type of cells studied	μ atoms of glucose carbon oxidized/ 5×10^8 cells	Inhibition %
Normal	—	Intraperitoneal	Thymus	3.54	—
	Hydrocortisone			2.30	35
	Deoxycorticosterone			2.64	25
Adrenalectomized	—	Subcutaneous	Thymus	3.51	
	Hydrocortisone			2.56	27
	17α-Ethyl-19-nortestosterone			3.69*	—
				4.11	+10
				3.77	+ 7
Normal	—	Intraperitoneal	Mesenteric lymph nodes	3.33	—
	Hydrocortisone			1.34	60
	Deoxycorticosterone			2.44	27
Adrenalectomized	—	Subcutaneous	Mesenteric lymph nodes	3.54	—
	Hydrocortisone			1.53	57
	17α-Ethyl-19-nortestosterone			1.57	56

* Each of these three values is the average of four experiments.

pensions from normal or adrenalectomized rats may be noted and indicates the reproducibility of the experiments. Hydrocortisone injection into normal or adrenalectomized rats produced an average inhibition of approximately 30% in glucose oxidation *in vitro* by thymic cells, and an inhibition of approximately 60% when mesenteric lymphoid cells were studied. Deoxycorticosterone injection produced an average inhibition of glucose oxidation of approximately 26% for both thymic and mesenteric cells.

The data indicate that whatever the mechanism may be of the in vitro *inhibitory effects of hydrocortisone and deoxycorticosterone on lymphocyte metabolism, similar metabolic influences are exerted by these steroids on the lymphoid tissue of the intact animal.* This is noteworthy particularly for deoxycorticosterone, which has been reported to be without influence on lymphoid tissue size or structure in the doses employed (1, 3). It appears that although deoxycorticosterone was a much more effective inhibitor than

hydrocortisone when added to cells *in vitro*, it was less effective when injected into the animal. This was also seen in experiments, not presented here, in which oxidation of labeled glucose by intact mesenteric lymph nodes was studied after injection of either hormone. Hydrocortisone produced a greater and more consistent inhibition than did deoxycorticosterone.

Subcutaneous injection of 17α-ethyl-19-nortestosterone produced a marked inhibition of glucose oxidation by cells of the *mesenteric lymph*

TABLE 4. EFFECTS OF VARIOUS STEROIDS ON ENDOGENOUS RESPIRATION, OXYGEN CONSUMPTION AND GLUCOSE OXIDATION OF SUSPENSIONS OF RAT THYMIC CELLS

(Each experiment conducted with air as gas phase at 37° C for 3 hr. Values given are % inhibition produced by 180 μg. of each steroid.)

Steroid	Glucose oxidation	Oxygen consumption*	Endogenous respiration
Cortisone	20	14	26
Cortisone-21-acetate	26	5	6
Dihydrocortisone-21-acetate	5	9	0
Tetrahydrocortisone-21-acetate	11	5	14
Δ^1-Cortisone	13	12	12
Hydrocortisone	31	32	16
Hydrocortisone-21-acetate	30	13	8
Δ^1-Hydrocortisone	41	32	12
9-α-Fluorohydrocortisone	30	16	20
6-Methyl-Δ^1-Hydrocortisone	27	17	10
11-Deoxycortisone	12	17	23
11-Deoxycortisone-21-acetate	2	2	3
Corticosterone	68	63	31
Deoxycorticosterone	93	86	46
Deoxycorticosterone acetate	39	40	21
Progesterone	74	65	42
17α-Hydroxyprogesterone	9	11	8
17α-Acetoxyprogesterone	2	2	0
Testosterone	30	29	31
Dihydrotestosterone	43	33	28
17α-Ethyl-19-nortestosterone	99	94	0
17α-Ethyltestosterone	97	88	87
19-Nortestosterone	93	77	65
Estradiol	33	21	15
Cholesterol	5	2	2

* With added glucose.

nodes (Table 3). However, the effect of this steroid on glucose oxidation by *thymic cells* was shown to be one of slightly augmenting oxidation. This remains to be investigated further.

In Vitro Influence of Steroid Structure on Oxidation of Glucose and Respiration by Thymic Cell Suspensions

Cell suspensions of rat thymus glands were employed in this study because of their ease of preparation in uniform quantity. Each steroid was studied at a single added quantity, *viz.*, 180 μg. Incubations were for 3 hrs. at 37° C with air as the gas phase. The data obtained are assembled in Table 4. Effects varying less than 10% from the controls are not considered significant. All of the significant changes observed by an effective steroid

were of an inhibitory nature, *i.e.*, a diminution in the control levels of glucose oxidation, oxygen consumption, and endogenous respiration.

Several steroids are characterized by a strikingly high degree of inhibition of glucose oxidation and oxygen consumption by lymphocytes. These are, in approximately decreasing order of effectiveness, 17α-ethyl-19-nortestosterone, 17α-ethyltestosterone, 19-nortestosterone, deoxycorticosterone and progesterone. It is apparent, therefore, that the dihydroxyacetone side-chain at C-17 is not essential for the inhibitory phenomena. Moreover, esterification of the C-21 hydroxyl group with acetate appears to diminish significantly the inhibitory activity of a steroid (compare deoxycorticosterone vs. deoxycorticosterone acetate; 11-deoxycortisone vs. 11-deoxycortisone acetate). This alteration in activity is sometimes more striking in terms of endogenous respiration and oxygen consumption in the presence of added glucose, than is the case with regard to glucose oxidation (compare cortisone vs. cortisone acetate; hydrocortisone vs. hydrocortisone-21-acetate). Notwithstanding, evidence that the 21-hydroxyl group is not essential for the inhibitory effects of the C-21 steroids is provided by the marked inhibitory activity of progesterone.

A strikingly uniform diminished inhibitory activity of C-21 steroids is observed when an α-hydroxyl group was present in the molecule at C-17. This is seen in comparisons of effectiveness of corticosterone vs. hydrocortisone, deoxycorticosterone vs. 11-deoxycortisone, deoxycorticosterone-21-acetate vs. 11-deoxycortisone-21-acetate and progesterone vs. 17α-hydroxyprogesterone. In the cortisone series, an 11-hydroxy compound produced a greater inhibition than did an 11-keto or 11-deoxy compound (compare hydrocortisone with cortisone and deoxycortisone; hydrocortisone acetate with cortisone acetate and 11-deoxycortisone acetate; Δ^1-hydrocortisone with Δ^1-cortisone). The reverse is true in the corticosterone series (compare corticosterone with deoxycorticosterone).

It may be noted from Table 4 that the diverse steroids which have been shown to have marked but varying degrees of glycogenic and anti-inflammatory actions have approximately the same effectiveness in inhibiting glucose oxidation. Thus, alterations in the molecule which significantly enhance glycogenic or anti-inflammatory effectiveness, e.g., introduction of a double bond between C-1 and C-2, or of a fluorine atom at C-9, or substitution of a methyl group at C-6, do not produce comparable enhancement of activity or even significant changes in the ability of a steroid to inhibit the glucose oxidation, endogenous respiration, or oxygen consumption of thymic lymphocyte suspensions.

In the series of C-21 compounds tested, saturation of ring A significantly reduced the inhibitory action of a steroid (*cf.*, di- and tetrahydrocortisone acetates). This was not the case, however, with the C-19 compounds, in view of the similar inhibitory activities of testosterone and dihydrotestosterone.

The exceptionally high inhibitory effectiveness of 17α-ethyl-19-nor-

testosterone, 17α-ethyltestosterone and 19-nortestosterone suggest a) that the angular methyl group at position 10 of the steroid nucleus is not essential for the activity observed, and b) a rather unique increase in activity in the C-19 series is attributable to the presence of an ethyl group at C-17. The activity of estradiol is of interest and suggests that an aromatic ring A may be substituted for the 3-ketone, $\Delta^{4,5}$, ring of C-19 and C-21 steroids without markedly altering the inhibitory effectiveness of a steroid. Indeed, the C-18 effective steroid may be a "nor" steroid, e.g., 19-nortestosterone, or may have an aromatic ring A, e.g., estradiol.

The data in Table 4 also reveal that several diverse steroids exhibited no significant activity under the experimental conditions employed.

DISCUSSION

The present investigation represents a portion of studies initiated to examine the biochemical basis for the profound alterations in lymphoid tissue histology seen shortly after the *in vivo* administration of certain adrenal cortical steroids (1–3). Two of these steroids *viz.*, cortisone and hydrocortisone, also produce changes in lymphocyte morphology when added to these cells *in vitro* (11–13), thus suggesting an *in vitro* study of possible biochemical alterations of lymphocyte metabolism in the presence of added steroid.

In view of the relatively specific requirements of structure of the 11-oxy adrenal cortical steroids for the production of lymphoid tissue involution *in vitro*, the data of the present study were somewhat unexpected. A wide variety of steroids, including C-18, C-19 and C-21 compounds, added *in vitro* to cell suspensions of lymphocytes, will alter the endogenous respiration of these cells, as well as their oxygen consumption in the presence of added glucose and their capacity to convert this glucose to carbon dioxide. The data indicate, moreover, that certain steroids may affect lymphocyte metabolism and yet be without discernible histological influence on lymphoid tissue. This appears to be the case, for example, with deoxycorticosterone. This compoind in the doses studied has been described to be without effect on lymphoid tissue size or structure when administered *in vivo* (2, 3). Nevertheless, deoxycorticosterone added *in vitro* to cell suspensions of normal or malignant lymphocytes profoundly depressed endogenous respiration, oxygen consumption in the presence of added glucose, and the oxidation of this glucose to carbon dioxide. Of greater significance is the fact that injection of deoxycorticosterone in rats led to alterations of thymic and mesenteric lymphocytes *in vivo* which were manifest in the *in vitro* studies of these cells obtained from the animals four hrs. after steroid injection. Thus, a steroid which does not alter thymic tissue histology when injected, had, nevertheless, induced biochemical changes in this lymphoid tissue which were demonstrable in thymic lymphocyte suspensions prepared from deoxycorticosterone-injected rats.

Also noteworthy is the fact that the steroids which caused an inhibition

in glucose oxidation were effective at concentrations of approximately 6.4×10^{-4} molar and less. This low concentration of steroid is approximately one half that required of cyanide in order to suppress lymphocyte respiration by that respiratory poison (18).

Inhibitory effects of a variety of steroids on carbohydrate oxidation by tissues *in vitro* have been reported. For example, Gordan and Elliot (30) found that a number of steroids, including deoxycorticosterone, inhibited the oxidation of glucose by broken cell preparations of rat brain. Bozonic, Laupin and Verzar (31) reported that glycogen synthesis from glucose by abdominal wall muscle *in vitro* was totally inhibited by addition of deoxycorticosterone. Grossman, Ryder and Pearson (32) have observed an inhibition of glucose utilization by rat diaphragm *in vitro* as a consequence of addition of hydrocortisone, both with respect to conversion to glycogen and oxidation to carbon dioxide. Deoxycorticosterone inhibited glycogen formation, but not the conversion of glucose to carbon dioxide.

The fact that each of the effective steroids produced a decrease in measured metabolic activities of lymphocytes may indicate that the inhibitions observed were a result of a common effect of these compounds on biochemical phenomena of basic importance in lymphocyte metabolism. One of these phenomena could be an altered permeability of the cell membrane. Alterations in cellular permeability have been proposed as a basis for one explanation of the metabolic effects of certain steroids (23, 33, 34). However, some of the data obtained suggest that at least a portion of the effects observed can be attributed to an action of the steroid upon processes within the lymphocyte, rather than due solely to action of the steroid upon the permeability of the lymphocyte membrane. Firstly, in specific instances, the *in vivo* injection of a steroid resulted subsequently in metabolic alterations measured *in vitro* in the lymphoid tissue of the injected animal, which were similar to those seen when the steroid was incubated directly with the lymphoid tissue *in vitro*. Secondly, a variety of steroids produced a marked inhibition of the *endogenous* respiration, in the absence of added substrate. Thirdly, steroids which were highly effective in the present study, e.g., 17α-ethyl-19-nortestosterone, deoxycorticosterone, and testosterone, are entirely without influence on the transaminase activity of thymic lymphocyte suspensions, whereas hydrocortisone markedly alters transaminase levels of thymic cells (28). It would appear therefore, that specific enzymatic processes within lymphocytes may be selectively affected by various steroids.[7] Hayano, Dorfman and Yamado (35) found that deoxycorticosterone and other steroids added *in vitro* influenced the activity of a variety of enzyme systems.

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[7] Unpublished data obtained since this manuscript was submitted for publication indicate that addition of deoxycorticosterone to a cellfree preparation from lymphocytes *in vitro* will inhibit glucose oxidation.

Some degree of specificity could be discerned in the steroid structure required for production of the effects observed in lymphocyte metabolism. The most striking structural feature seen in the C-21 series of compounds was the greater degree of activity of 17-deoxy, as compared to 17-hydroxy, compounds. Introduction of a 17-hydroxyl group into corticosterone, deoxycorticosterone, or progesterone markedly diminished the capacity of these compounds to depress glucose oxidation and oxygen consumption by thymic lymphocytes. Although esterification of the C-21 hydroxyl group decreased the activity, as compared to that of the parent steroid, this group is not essential for the effects observed inasmuch as progesterone proved to be one of the most potent compounds tested. Moreover, the dihydroxyacetone side chain at C-17 was not essential for the activities studied, since C-18 steroids (estradiol) and C-19 steroids (testosterone and its derivatives) were also effective under the conditions employed. Indeed, 17α-ethyl-19-nortestosterone proved to be the most potent steroid tested. The possible specificity of this effect is seen from the fact that this steroid is without influence on the transaminase activity of thymic lymphocytes *in vitro* (28). On the other hand, 17α-ethyl-19-nortestosterone does inhibit strikingly the incorporation of radioactivity into the total nucleic acids and proteins of lymphocyte suspensions incubated with glycine-2-C^{14} (18).

It may also be noted that while reduction of ring A in the C-21 series abolished the inhibitory effects of an active compound with respect to oxygen consumption and glucose oxidation, similar reduction in the C-19 series did not alter activity. Moreover, activity is still evident in compounds in which ring A is aromatic, e.g., estradiol. Finally, it may be pointed out that the presence of an α-ethyl group in the 17 position in the C-19 series led to compounds with the highest potency seen in this study.

Only two steroids, deoxycorticosterone and hydrocortisone, were examined in studies with cell suspensions prepared from the transplantable lymphosarcoma. These cells appeared to be more sensitive to the presence of hydrocortisone than did thymic lymphocyte suspensions. The same concentration of steroid produced an inhibition of 69% in glucose oxidation by lymphosarcoma cells, and only a 31% inhibition when thymic cells were studied. Similar relative data were seen in measurements of oxygen consumption. Deoxycorticosterone was a more effective inhibitor of the metabolism of lymphosarcoma cells than was hydrocortisone; it will be recalled that this was also true with thymic cell suspensions.

It may be concluded that *in vitro* effects of steroids on the metabolism of normal and malignant lymphocyte cell suspensions have been demonstrated. Evidence has been obtained to support the suggestion that these effects have some degree of specificity, both with respect to the nature of the reactions in lymphocytes which are altered, and the structural characteristics required in the steroid molecule for manifestations of these activities.

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THE PHOTOCHEMICAL ACTIVATION OF THYROXINE

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ABSTRACT

Recent evidence suggests that thyroxine is not the active form of the thyroid hormone. Since thyroxine's phenolic hydroxyl group appears to be quite labile, changes in this group may be involved in converting thyroxine to its active form. Light was found to catalyze an apparent oxidation of the hydroxyl group, and this photosensitivity suggested the possibility that exposure to light might hasten thyroxine activation. Accordingly, Q_{O_2} was measured in liver slices and in diaphragm from normal and thyroxine injected rats, with half the tissues in each group exposed to brilliant illumination. Both tissues exhibited an increased response to thyroxine when subjected to the light, although the effect on liver was considerably greater than on muscle. It is postulated that thyroxine activation may require oxidation of the phenolic hydroxyl group and that a free radical may represent the active form of this hormone.

ALTHOUGH thyroxine is now accepted as the principal circulating form of the thyroid hormone, a number of investigators have suggested that it must be converted to an active form by responding peripheral tissues before its metabolic effect is manifested. Among the possible molecular changes which might be associated with this conversion, the oxidation of the phenolic hydroxyl group merits particular consideration. Niemann, *et al.* (1, 2) and Gemmill (3) have suggested that such an oxidation may be involved in the activity of thyroid hormone. Our attention also was directed to the hydroxyl group when we observed that the extinction coefficient for thyroxine's absorption maximum in the ultraviolet (at 325 mμ) is altered during exposure to light. Ordinary laboratory illumination was found to induce a considerable increase in extinction coefficient over the course of several days, but this effect was ultimately reversed as visible pigmentation developed. This process, undoubtedly an oxidation, was accelerated by intense illumination (particularly by ultraviolet light) and inhibited by darkness. The ultimate pale yellow color is apparently caused by formation of quinones. Contrary to once commonly held belief, it is not caused by the liberation of free iodine; no free iodine could be detected. Moreover, the observed increase in optical density is incompatible with deiodination in that the probable products (triiodothyronine and diiodothyronine) have lower extinction coefficients than thyroxine in the spectral region studied.

Since the observed spectrophotometric changes demonstrate that thyroxine is photosensitive and also suggest that the phenolic hydroxyl group

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is quite labile, it seemed reasonable to consider the possibility that oxidation of this group is involved in the conversion of thyroxine to an active form. Thus, operating on the hypothesis that tissues *slowly* accomplish this conversion, the experiments herein reported were designed as attempts to catalyze the formation of an active derivative of thyroxine through the use of light.

MATERIALS AND METHODS

Male Sprague-Dawley rats provided the tissues used; diaphragms were taken from weanling animals weighing about 60 gm.; livers, sliced about 0.3 mm. thick, were taken from adults. Half the animals in each group received a single 5 mg. subcutaneous dose of *d,l*-thyroxine about 3 hours prior to being sacrificed. The rate of oxygen consumption of the isolated tissues was measured over a 5 hour period at 38° C by the direct Warburg method, using a phosphate buffered glucose-Ringer's solution previously described (4). Each flask was initially gassed with oxygen for about 5 minutes, and readings were taken at half hour intervals. Over the entire 5 hours, half the pre-thyroxinized and control tissues were subjected to intense illumination provided by a battery of 4 fifteen watt fluorescent tubes (white) submerged in the water bath as close as possible to the oscillating vessels. Tissue temperature, measured with thermocouples, was found not to be influenced by this system of illumination. Thus, Q_{O_2} values were obtained for 4 different groups of liver and diaphragm: 1. unilluminated controls, 2. illuminated controls, 3. unilluminated thyroxine pretreated, and 4. illuminated thyroxine pretreated tissues. Statistical evaluations were made by analyses of variance.

RESULTS

As indicated in Figure 1, thyroxine alone exerted a measurable influence on the Q_{O_2} of diaphragm, depressing it for the first 1.5 hours ($P < 0.05$) and having a reverse effect during the last 1.5 hours ($P < 0.01$). This is in accord with the recent work of Weinstein and Lein (4) who demonstrated a similar effect of *in vitro* thyroxine on diaphragm. Illumination alone seemed to elevate the Q_{O_2} of diaphragm but had a considerably greater effect on those tissues taken from thyroxine pretreated animals ($P \cong 0.05$).

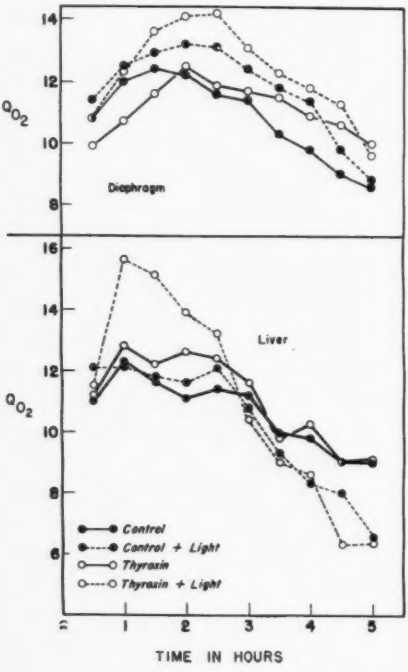

FIG. 1. The Q_{O_2} (dry weight) of illuminated and unilluminated diaphragm and liver slices from control and thyroxine pretreated rats, measured over a 5 hour period. Each point represents the mean of 9 determinations.

In the case of liver slices, illumination alone had no measureable influence on Q_{O_2} during the first few hours. However, after the third hour, Q_{O_2} values for illuminated controls decreased more rapidly than those of the unilluminated controls so that by the fifth hour there was a significant difference ($P < 0.01$) between them. In unilluminated tissues, pretreatment with thyroxine exerted a small effect ($P < 0.05$) only at about the second hour.

The effect of illumination on the thyroxine pretreated liver slices was rapid and dramatic. The Q_{O_2} rose rapidly, reaching a peak well over 20% above the mean Q_{O_2} for unilluminated thyroxine pretreated tissues at the first hour ($P < 0.01$). After that time, however, there was a relatively rapid decrease so that by the fifth hour the Q_{O_2} was similar to that of the illuminated control group and significantly lower than that of the unilluminated groups ($P < 0.01$).

The Q_{O_2} values presented are based on the dry weight of the tissues at the conclusion of the experiments, and since all the tissues lose weight at approximately the same rate regardless of experimental group (4), the Q_{O_2} values for the first few hours are overestimated and the final rate of decline is not as great as indicated in the graphs.

DISCUSSION

The metabolic response of unthyroxinized diaphragm to light suggests that this tissue normally possesses photosensitive materials or systems apparently absent in liver. This finding, of course, somewhat complicates the detection of an effect of light on the response to thyroxine by diaphragm. However, as can be seen in Figure 1, diaphragm's response to light was greater when thyroxine had previously been administered, and the response to thyroxine was enhanced by illumination. An analysis of variance of the Q_{O_2} values from the first through the third hour of the experiments disclosed significant interaction ($P \cong 0.05$) between the effects of light and thyroxine.

This complicating feature does not pertain to liver slices since there was no early response to light alone, and the effect of light in potentiating the response to thyroxine is in this case obvious and unequivocal. The liver's capacity for rapidly accumulating injected thyroxine (5, 6, 7) may account in part for the more obvious response by this tissue.

Although the results of the experiments reported here are subject to numerous interpretations, they support the contention that thyroxine is not the active form of the thyroid hormone and further suggest that an oxidation product of thyroxine may be the active form (8). A number of attempts were made to elicit a rapid metabolic response with the stable products of oxidation (preirradiated thyroxine solutions). These were uniformly unsuccessful. Thus, it seems likely that an intermediate compound may be important. The oxidation of thyroxine probably involves the for-

mation of an intermediate free radical (a semi-quinone) which is highly unstable in ordinary solutions in the physiological pH range. It is postulated that this free radical, possibly stabilized by the intracellular protein(s) with which it interacts (9), is the active form of the thyroid hormone. It should be mentioned in this connection that Gemmill (3) has suggested the formation of a free radical in the action of thyroxine on the ascorbic acid-ascorbic acid oxidase system and that a similar free radical (of diiodotyrosine) has been postulated (10, 11) in the biosynthesis of thyroxine. In addition, Frieden and Flitman (12) have discussed the importance of an "oxidized thyroxine".

Vind, *et al.* (13) have prepared sulfonate esters related to thyroxine, and the potency of these was found to increase on exposure to sunlight. This activation, according to the authors, is caused by the re-establishment of the normal diphenyl ether configuration and thus is probably not related to the effects described here. It is of interest to note, however, Vind's finding that when substitution of the thyroxine phenolic hydroxyl group with an amino group was presumably achieved, all thyromimetic activity was lost.

In a provocative series of papers, Thibault (14–16) and Pitt-Rivers (17, 18) have reported the action of acetic acid derivatives of thyroxine (Tetrac) and triiodothyronine (Triac) on intact animals and on isolated tissues. Their results indicate that both of these compounds elicit a rapid metabolic response wherein the usual latent period characteristic of the response to thyroxine (T4) and triiodothyronine (T3) is minimized or absent. They have further reported (19, 20) that when kidney or liver slices are incubated at 4°C in the presence of T3 or T4, there is a partial conversion to rapidly acting derivatives. Roche and his collaborators (21–25) have shown that T3 is converted to Triac in the kidneys and in muscle of the rat, while Tomita, *et al.* (26) find that kidney mitochondrial enzymes are able to convert T3 and T4 to Triac and Tetrac respectively.

In the light of their results, Thibault and Roche and his collaborators have postulated that Triac and Tetrac are active forms of the thyroid hormone. The experimental results presented in this report do not contradict their proposal since the chemical identity of the "photoactivated thyroxine" has not been established conclusively. However, Barker (27, 28) finds that not only are Tetrac and Triac somewhat less potent than T4 and T3 respectively but that they also exhibit latent periods approximately equal to those of the parent compounds. Barac (29) also fails to find an immediate metabolic response to Tetrac.

Thibault has pointed out that Triac and Tetrac induce a rapid metabolic response at a low dose or concentration but at higher levels they may become ineffective or even cause a reduction in metabolism. Thus, Thibault's experiences with Triac and Tetrac are reminiscent of similar findings reported for thyroxine by other investigators (4, 30–34). This then suggests the likelihood that although Tetrac, Triac, T3, and T4 are all naturally occurring forms of thyroid hormone, none of them may be active form

but, to the contrary, may at sufficiently high concentration reduce metabolic rate by competitively inhibiting the active hormone.

A large number of thyroxine analogues involving modifications of the alanine portion of the T4 molecule has been studied, and many of these have been found to possess thyromimetic activity (35). In those cases, however, where the phenolic oxygen has been removed (13, 35), thyromimetic activity is usually lost. In fact, many such compounds are T4 inhibitors. Thus, in light of the arguments presented, it is tempting to postulate that changes in the aliphatic side chain may serve primarily to alter the ease of penetration of the cell or the strength of the bond between the hormone and its action site but that oxidation of the phenolic hydroxyl group is required for activation.

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SOME PROPERTIES OF AN OXYTOCIC SUBSTANCE FOUND IN BLOOD EXTRACTS

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ABSTRACT

Some physico-chemical and pharmacological properties of an oxytocic substance (O.S.) in acid-alcohol extracts of human blood have been investigated. This substance is not believed to be oxytocin, K^+, acetylcholine, H.T., histamine, hypertensin, substance P, pepsitocin, or P.P.S. It is probably a polypeptide and of the polypeptides which are known to stimulate smooth muscle, it most closely resembles bradykinin, kallidin, or substances U and Z.

AN OXYTOCIC (uterine-stimulating) substance (O.S.) can be extracted from blood. It does not appear to be oxytocic hormone. This substance has been found in blood extracts of (a) non-pregnant women and women during normal pregnancy and parturition (1); (b) lactating and non-lactating women (2 and 3); lactating cows and goats (4); and in sheep and the human male. This material (or a substance closely resembling it) has been extracted from the hypothalamus of cats, rats, mice and cows (5), and was not found in extracts of other parts of the brain and spinal cord. The behavior of hypothalamic extracts will be reported in a subsequent paper.

The essential test used to distinguish this substance from oxytocin is treatment with thioglycollic acid (6 and 7) which inactivates oxytocin but not O.S.

Many substances will stimulate the rat uterus to contract, and besides oxytocin these include potassium, acetylcholine, 5-hydroxytryptamine (HT), hypertensin (angiotonin), vasopressin (antidiuretic hormone), pepsanurin, pepsitocin, pepsitensin, bradykinin, kallidin, substance P, substance Z, substance U and pain-producing substance (P.P.S.).

This paper reports some of the physico-chemical and pharmacological properties of an unidentified oxytocic substance in blood extracts.

METHODS

Preparation of the extracts

Samples of blood were collected from the antecubital veins of healty non-pregnant medical students and prevented from clotting by adding heparin. The bloods were added immediately to acid-alcohol and extracted according to the authors' modification

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1) of the method of Bisset and Walker (8). The possibility of 5-hydroxytryptamine or acetycholine in the extracts acting as uterine-stimulating substances was removed by dialysis and/or by adding Dibenamine hydrochloride and atropine to the organ bath. After dialysis through a cellophane membrane the concentration of potassium in the extracts was estimated directly by a flame photometer, and the amount of this ion contained in the largest doses of extract used was compared with the uterine threshold dose of potassium. In this way 5-HT, acetylcholine and K^+ were eliminated as interfering oxytocic substances in these experiments. A sample of each extract (or in some instances the whole extract) was treated with sodium thioglycollate (0.01 *M* at pH 7.5 for 30 minutes) in order to inactivate oxytocin (6 and 7).

Methods of assay

(a) *Rat uterus.* Extracts were assayed for oxytocic activity (O.A.) against Pitocin (Parke, Davis and Co.) on the isolated sensitized uterus of virgin Wistar strain rats as described by Holton (9), and Gaddum, Peart and Vogt (10).

(b) *Isolated rabbit and guinea pig intestine.* Rabbits and guinea pigs were killed by a blow on the back of the head. A 3–4 cm. portion of the upper ileum was suspended, after emptying its lumen, in a 10 ml. organ bath in Mg^{++}-free Tyrode's solution containing 0.10% $NaHCO_3$, and through which O_2 was bubbled. Contractions of only the longitudinal muscle were recorded with a frontal writing lever which had a five fold magnification.

(c) *Mammary gland of lactating rabbit.* Rabbits with kittens 7–14 days old were used and they were isolated from their young overnight. The next morning they were anaesthetized with 70 mg pentobarbitone/kg. About 1–2 mm. was cut from tip of one nipple and a gauge 22 hypodermic needle (specially blunted) was introduced into a lactiferous duct and securely tied. To this was attached a small length of polythene tubing of 1 mm. internal bore which was in turn connected with upright glass tubing. The needle and polythene tube were filled with 1% sodium citrate the meniscus of which in the glass vessel was about 2 cm. above teat level. All injections (at pH 7 to 7.5) were made through a needle inserted in an ear vein. The method was sensitive to 0.5 mU. oxytocin (as Pitocin) and would discriminate 25% increments in doses with reproducible precision.

(d) *Rat blood pressure.* Male Wistar strain rats weighing about 300 gm. were anaesthetized with urethane (175 mg./100 gm. body weight) injected subcutaneously. About 60 min. later a polythene cannula was inserted into the right jugular vein. A cannula was tied in the left carotid artery and this was connected with a special mercury manometer (11) of 3 mm. internal diameter by a column of normal saline. Heparin (200 U. per 100 gm. body weight) was injected through the venous cannula and washed in with saline.

Stability on boiling at various pH

Aliquots of these extracts were boiled in a water bath with condenser as follows: 60 min. at pH 1; 60 min. at pH 7.5; 5 min. and 7.5 min. at pH 0.2; 5 min. and 7.5 min. at pH 11.5. Control samples of the extracts were kept at room temperature at similar pH to the experimental aliquots. Immediately after boiling the samples were cooled by ice and the pH adjusted to 7.5. A part of each extract was kept as control at pH 7.5.

Hydrolysis with enzymes

The enzymes used were pepsin, trypsin, chymotrypsin and carboxypeptidase. To 3.0 ml. samples of extract 2.0 mg., 4.0 mg. and 8.0 mg. of either enzyme were added and for pepsin, the pH was adjusted to 2.5 and for the others to 7.3. These, together with control aliquots of extract, were incubated at 37° C for 6 hours. Each experiment was repeated 4 times. Solutions containing 20 mU. of Pitocin were treated similarly.

Pregnancy plasma contains an enzyme which inactivates oxytocin (and vasopressin)

(12). To 3.0 ml. samples of extract 0.1 ml. of pregnancy plasma was added and incubated at pH 7.5 for 2 hours. The same quantity of plasma was added to 100 mU. Pitocin and incubated under similar conditions.

To test extract for presence of pain-producing substance (P.P.S.)

An aliquot of the extract (with an original pH of 7.5) was boiled for 30 min. at 100° C and pH 0.2 so as to destroy O.S. The pH was then corrected to 7.5. This extract and the control were separately (0.05 ml.) injected intradermally into the flexor surfaces of the forearms of 8 subjects who subjectively appraised the intensity of the pain produced by the extracts.

Drugs

All drug doses given in the text refer to the salts. These were nicotine acid tartrate, atropine sulphate, histamine acid phosphate, mepyramine maleate, acetylcholine chloride, 5-hydroxytryptamine creatinine phosphate, adrenaline hydrochloride and Dibenamine hydrochloride.

RESULTS

Assays on isolated rat uterus and rabbit and guinea pig intestine. The rat uterus was sensitive to 0.2 mU. Pitocin (and synthetic oxytocin), the rabbit ileum to 75–100 mU. Pitocin, whilst the guinea pig ileum was insensitive to 300 mU. doses. The guinea pig ileum was sensitive to 100 ng HT; 0.1 ml. 4×10^{-8} histamine; 0.1 ml. 10^{-5} nicotine; 0.2 ml. 10^{-8} acetyl choline; the rabbit ileum contracted to 50 ng HT; 0.01 ml. 4×10^{-2} histamine; 0.1 ml. 10^{-5} nicotine; 0.1 ml. 10^{-8} acetylcholine; 0.13 ml. 4% KCl; the rat uterus responded to 5 ng HT and 0.2 ml. 10^{-8} acetyl choline. In each case appropriate antagonists of the above were used. Each preparation was insensitive to adrenaline and thioglycollic acid, and in the case of the uterus to histamine. The threshold dose of extract on each preparation was 0.3–0.4 ml. and the effect of the extract could not be blocked by atropine, mepyramine, Dibenamine or lysergic acid diethylamide.

Assays on mammary gland of lactating rabbit. Two concentrated blood extracts were used which had a potency of 40 and 18 mU./ml. oxytocic activity when tested on the rat uterus against both synthetic oxytocin and Pitocin. No measurable change in intra-mammary pressure was recorded when doses up to 1.0 ml. of each extract were given, although pressure changes were produced by 0.5 mU. oxytocin and Pitocin.

Assays on rat blood pressure. All injections were made through the jugular cannula and washed in with 0.9% sodium chloride; the pH of the extracts was corrected if necessary to between 7.0 and 7.5 before injecting. Five active extracts (as tested in parallel on the rat uterus) were used and these caused a rapid and transient fall in the blood pressure, which was sometimes followed by a slight rise but the effect was over within 1 to 2 min. One extract which was inactive when assayed on the rat uterus gave a similar rapid 5–10 sec. transient vasodepressor effect. After boiling the 5 active extracts for 60 min. at pH 1 (a procedure which does not destroy O.S.) and then injecting them, there were no changes in the blood pressure;

TABLE 1. STABILITY OF OXYTOCIC SUBSTANCE TO TEMPERATURE AND PH

Preparation	pH	Temp. (C)	Min. exposed to temp.	% inactivation
(1) & (2)	1.0	100	60	0
	1.0	25	60	0
	7.5	25	60	0
(3)	0.2	100	7.5	86
	0.2	24	7.5	86
	7.5	24	7.5	0
(4)	0.2	100	5.0	84
	0.2	24	5.0	78
	7.5	24	5.0	0
(5)	11.5	100	5.0	43
	11.5	24	5.0	25
	7.5	24	5.0	0
(6)	13.0	100	7.5	92
	7.5	24	7.5	0
(7)	7.5	100	60	74
	7.5	24	60	0

yet, when the same extracts were boiled at pH 0.2 for 30 min. (to destroy O.S.) and then administered they gave definite pressor responses which were maintained for about 2 min.

Stability on boiling at various pH. Reference to Table 1 shows that oxytocic substance resists boiling at pH 1.0 but is rapidly inactivated at room temperature in highly acid and alkaline solutions. On boiling at pH 7.5 for 60 min. there was 74% loss of activity.

Hydrolysis with enzymes. There was no loss of activity in either blood extracts or Pitocin solutions after incubation at 37° C under optimal pH conditions with trypsin or pepsin (Table 2). With chymotrypsin, however, there was substantial loss of activity in both blood extracts and standard Pitocin, whilst carboxypeptidase destroyed O.S. but not Pitocin.

Is pain-producing substance in the extracts? In order to test for the presence of P.P.S. in extracts the following procedure was adopted. A needle was placed in the antecubital vein of a healthy subject and blood was drawn into a silicon coated glass syringe and then extracted using a combination of silicone coated glassware and polythene vessels so that there was no contact with glass during the extraction or during the assay. Blood collected through the same needle into a glass syringe and extracted with glass ap-

TABLE 2. EFFECT OF ENZYMES ON OXYTOCIN AND OXYTOCIC SUBSTANCE

Preparation	Inactivated by			
	Chymotrypsin	Carboxypeptidase	Pepsin	Trypsin
Oxytocin	+	−	−	−
Oxytocic substance	+	+	−	−

paratus served as a control. This experiment was repeated 4 times using different subjects. In 2 experiments the extracts were assayed 4 to 6 hr. after the extracts were prepared, and in the other 2 experiments the assays were made on the following day after storing at −20° C in the deep freeze. The extracts were treated with thioglycollate in each instance, and when assayed on the rat uterus no significant differences in the oxytocic activity of the 2 samples of the same blood could be detected. When aliquots of these extracts were boiled at pH 0.2 for 5 to 7 min. and tested on the rat uterus they were found to have lost oxytocic activity. On readjusting the pH to 7.5 and injecting intradermally into 8 subjects the subjective sensations of pain were greater than in the control injections at the same pH. There was no itch associated with the pain, which after a latency of about 30 sec. increased in intensity up to about 2 min. and then quickly subsided.

DISCUSSION

As a result of these investigations it appears that the oxytocic substance in our extracts was not oxytocin. The evidence for this conclusion is that 1) O.S. is not inactivated by thioglycollic acid; 2) O.S. is retained during dialysis by a cellophane membrane through which oxytocin passes (5); 3) O.S. does not initiate the let-down of milk; 4) it stimulates the guinea pig and rabbit ilea which are relatively insensitive to oxytocin; and 5) it is destroyed by carboxypeptidase which does not inactivate oxytocin. On the other hand, however, O.S. resembles oxytocin because it is destroyed by chymotrypsin and pregnancy plasma and not by trypsin or pepsin; its dose-response curve on the virgin rat uterus is not significantly different from that of Pitocin (5); it is easily destroyed by alkalis or strong acids.

The results of the assays using the rat uterus and guinea pig and rabbit intestine also show that O.S. is not confused with acetylcholine, HT, histamine, adrenaline or K, and which confirms our earlier experiments (1). A useful finding was that O.S. is almost equiactive on the rat uterus, and the guinea pig or rabbit ileum. Since these preparations are reported to be sensitive to pain-producing substance (13) and to bradykinin (14, 15) the possibility emerges that O.S. might be P.P.S., or bradykinin or a polypeptide with similar actions, e.g. kallidin, substance U or substance Z. Since these were not available it was not possible to test them on the assay preparations.

A number of polypeptides which stimulate smooth muscle have been described and the effects of enzymes upon them (16) have been explained.

We have also found that oxytocin is not inactivated by trypsin or pepsin —thus confirming a view held by Croxatto (17). Reference to Table 3 shows that pepsitocin and substance Z also resist hydrolysis by trypsin and pepsin, and that kallidin, bradykinin and substance U are not inactivated by trypsin (the effects of pepsin on these 3 substances have not been clearly elucidated). Table 3 also reveals that chymotrypsin destroys the

activity of all the above polypeptides. On the basis of our experiments with chymotrypsin, carboxypeptidase, trypsin and pepsin it can be confidently concluded that oxytocic substance is most probably a polypeptide and that it is not hypertensin, pepsitensin, substance P, vasopressin or pepsanurin. If it is one of the polypeptides listed in this table, it is most likely to be oxytocin, pepsitocin, kallidin, bradykinin, substance U or substance Z. For reasons previously given oxytocin can be eliminated from this group.

Pepsitocin is prepared by acid or enzyme hydrolysis of plasma proteins (17). As with oxytocin it is not destroyed by trypsin, pepsin or carboxy-

TABLE 3. SOME PROPERTIES OF ACTIVE POLYPEPTIDES

	Effect on blood-pressure	Other actions	Formed by	Inactivated by		
				Chymo-trypsin	Tryp-sin	Pepsin
1. Vasopressin	Rise	Antidiuretic	—	+	+	0
Pepsanurin	0	Antidiuretic	Pepsin	+	+	0
2. Oxytocin	Fall	Contracts uterus	—	+	0	0
Pepsitocin	0	Contracts uterus	Pepsin	+	0	0
3. Cholecystokinin	—	Contracts gall bladder	—	—	—	—
4. Substance P	Fall	Contracts intestine	—	+	+	+
5. Kallidin	Fall	Contracts uterus and intestine	Kallikrein	+	0	—
Bradykinin	Fall	Contracts uterus and intestine	Trypsin, etc.	+	0	—
Substance U	Fall	Contracts uterus and intestine	Urine	+	0	—
Substance Z	Fall	Contracts uterus and intestine		+	0	0
6. Hypertensin (or angiotonin)	Rise	—	Renin	+	+	+
Pepsitensin	Rise	—	Pepsin	+	+	+

peptidase but is inactivated by chymotrypsin (17). Although relatively little progress has been made in the purification of pepsitocin (and pepsitensin and pepsanurin) there is evidence that they constitute 3 chemically distinct polypeptides. Since in our experiments the O.A. of the extracts was destroyed by carboxypeptidase it seems that this activity is not due to pepsitocin. In recovery experiments which are not reported here, it appears that O.S. is not an artifact formed during the preparation of the extracts.

Kallidin, bradykinin and substances U and Z are reported to have a transient vasodepressor effect (18). Our extracts had similar depressor effects on the blood pressure in urethane anaesthetized rats, and in one instance this occurred even when the extract had lost its oxytocic activity as assayed on the rat uterus. These experiments on rat blood pressure were not very informative as the extracts apparently contained interfering vasodepressor substances, but two interesting observations were made: after boiling the extracts at pH 1.0 for 60 min. there was no effect on the blood

pressure (i.e., the vasodepressor substances were destroyed but O.S. as tested on the rat uterus, was active) and after boiling for 30 min. at pH 0.2 (to destroy O.S.) the extracts consistently gave pressor responses.

Pain-producing substance is formed when blood contacts glass (13 and 19) and is thus apparently not free in the circulation. As well as producing pain, it is also an oxytocic substance in some species. Other pain-producing and uterine stimulating substances are acetylcholine, K, histamine, adenosine triphosphate, HT, bradykinin, angiotonin and leukotaxine.

The possibility therefore exists that O.S. and P.P.S. are the same substance. No difference was found however, in the O.A. of blood taken from the same donor at virtually the same time into silicone coated-polythene apparatus on the one hand, and into all glassware on the other. Furthermore O.S. is stable at −20° C for at least 4 months at pH 7.5 or pH 2.0 (and certainly for as many days at room temperature) whereas P.P.S. is rapidly inactivated at room temperature. It seems also that P.P.S. is destroyed by proteolytic enzymes (17). Also the extracts possess P.P.S. activity after the removal of the oxytocic effect of O.S. by boiling at pH 0.2.

Although identification of the oxytocic substance in our extracts is not complete, the evidence suggests that, of the known polypeptides which stimulate smooth muscle, it most closely resembles bradykinin, kallidin, substance U or substance Z.

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NOTES AND COMMENTS

HORMONAL EFFECTS ON COLLAGEN FORMATION IN GRANULOMAS[1]

ABSTRACT

Adrenalectomy, deoxycorticosterone and stilbestrol enhanced collagen formation in the carrageenan granuloma in guinea pigs; cortisone or corticotrophin tended to inhibit collagen formation; progesterone or testosterone had no demonstrable effect.

Although changes in connective tissues have been induced by a variety of hormones (1, 2, 3, 4) there have been few chemical assays of tissue constituents. There should be for such studies, either a relatively large amount of tissue or the substance assayed must undergo considerable change. We have demonstrated that collagen (5) and mucopolysaccharide formation (6) in the massive carrageenan (Irish moss) granuloma of guinea pigs varied with changes in dietary ascorbic acid. A similar sensitivity of this rapidly forming tissue to some endocrine influences is described here.

MATERIALS AND METHODS

Granulomas were induced in male guinea pigs, as described previously (5). Except as noted below, the animals weighed between 275 and 325 gms. A Macdonald #5 scorbutigenic diet (7), supplemented with 50 mg. of ascorbic acid every other day, was fed. After 14 days the granulomas were removed, freed of muscle and necrotic areas, and minced. Aliquots were analyzed for collagen by the method of Lowry *et al.* (8), and for water content by drying overnight at 105° C. Neutral fat was determined as the weight lost after extraction with ether and petroleum ether.

All hormones were administered subcutaneously at 12 hour intervals during the 14 days of granuloma development. Cortisone and corticotrophin were dissolved or suspended in saline; all other hormones were dissolved in sesame oil.

Adrenalectomy was carried out in two stages (the left adrenal was removed about 2 weeks after the right adrenal) and the animals were supported with daily subcutaneous injections of 0.5 mg. deoxycorticosterone acetate for about 3 weeks before inducing granulomas. The guinea pigs which then weighed 450–500 gm. did not withstand the trauma of carrageenan injection well unless supported for the succeeding 4 days. Even with this precaution less than half of the animals lived for 14 days after injection of carrageenan. Blood drawn from the hearts of survivors just prior to killing exhibited low serum sodium concentrations (119–136 meq./L), high serum potassium concentrations (5.8–9.0 meq./L), high N.P.N. (39–113 mg.%) and hemo-concentration, typical of adrenal insufficiency.

Controls for this study included a series of 16 small guinea pigs (250–350 gm.) and a series of 6 large guinea pigs (450–550 gm.) in which granulomas were induced but which received no other treatment; and a series of 6 animals which in addition were injected subcutaneously twice daily with 0.5 ml. sesame oil.

RESULTS AND DISCUSSION

The results are summarized in Table 1.

These data demonstrate hormonal effects on carrageenan granulomas in guinea pigs. A decreased collagen concentration was observed in granulomas of guinea pigs treated with cortisone or corticotropin but this reduction varied considerably ranging from collagen concentrations as low as 6% to normal concentrations of 12%. The observed decrease is in general agreement with the well documented inhibitory effect of 11-oxysteroids on connective tissue formation. Although this effect has been consistently ob-

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served in mice, rats and rabbits, previous reports have failed to demonstrate it in guinea pigs (9, 10).

The effect of deoxycorticosterone on connective tissue seems in general to be opposite to that of cortisone. Thus, enhancement of granuloma formation by deoxycorticosterone has been observed in the rat by Taubenhaus and Amromin (11), in the mouse by Schiller (12) and in the guinea pig by Pirani *et al.* (13). However, both Pirani and Taubenhaus reported a decrease in the amount of histologically demonstrable collagen in such granulomas. The increase in collagen content of granulomas was marked and consistent in animals treated with deoxycorticosterone in the present study. Though no measurements were made, the amount of granuloma also appeared to be increased.

Adrenalectomy has been reported to have only slight or no effect on granuloma for-

TABLE 1. EFFECT OF HORMONE TREATMENT ON COLLAGEN CONCENTRATION OF CARRAGEENAN-INDUCED GRANULOMAS

Treatment	No.	Collagen*	Water*
Normal	16	11.6 ± 0.6	660 ± 12
Normal, 0.5 ml. sesame oil injected subcut. 2× per day	6	11.7 ± 0.6	680 ± 13
Deoxycorticosterone, 5 mg. per day	5	15.0 ± 0.7**	670 ± 17
Stilbestrol, 0.5 mg. per day	6	15.0 ± 0.5**	660 ± 8
Progesterone, 2.5 mg. per day	6	12.8 ± 0.6	720 ± 18
Testosterone, 2.5 mg. per day	6	10.5 ± 0.6	638 ± 32
Normal large animals	6	10.3 ± 0.6	620 ± 26
Adrenalectomy	6	15.4 ± 0.6**	638 ± 31
Adrenalectomy. Cortisone, 2 mg. per day	7	9.8 ± 1.1	608 ± 37
Cortisone, 5 or 10 mg. per day	9	8.7 ± 0.8**	702 ± 18
ACTH, 4 or 8 units per day	9	9.6 ± 0.8	732 ± 18

* Grams per 100 gm. dry, fat-free tissue (mean ± standard error).
** Differ significantly from the normal $P < .01$.
The granulomas developed for 14 days after the subcutaneous injection of a suspension of 1% carrageenan and 1% sulfadiazine in saline. The hormonal treatment was carried out for the entire 14 days.

mation in rats (15), but no comparable studies have been reported for guinea pigs. The increased collagen concentration observed in our experiments may have been caused by the deoxycorticosterone, which it was necessary to administer for 4 days following injection of carrageenan, but more likely, it reflects the absence of the inhibitory 11-oxysteroids.

The increased collagen concentration following stilbestrol administration is also reported against a background of conflicting literature. Kosdoba (16) and others have observed improved healing of wounds in guinea pigs treated with estrogen, whereas Taubenhaus and Amromin (12, 14) reported that estrogen inhibited granuloma formation in rats.

The present results, as pointed out above, often conflict with reports in the literature based on histologic descriptions. This lack of agreement is probably due to several causes: (a) determination of the magnitude of histologic change is difficult; (b) there are species differences in reactivity to hormones; and (c) molecular collagen assayed in our experiments is not necessarily identical with the stainable morphologic entity. (For discussion see Robertson and Hinds (6).)

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GLUCAGON ADMINISTRATION IN PREGNANT RATS[1]

ABSTRACT

The effects of the administration of a total dose of 0.50 mg. of glucagon per day in pregnant and nonpregnant rats are reported. Alterations in transaminase activities, blood amino nitrogen, liver weight and body weight were produced. A significant decrease in the viable fetus weight and an increase in number of fetal deaths was seen in the glucagon treated rats. Although pregnant rats have been shown to exhibit a resistance toward the effects of cortisone, no such resistance is evident with regard to glucagon administration.

It has been reported that pregnant rats exhibit a resistance to the biochemical effects of cortisone administration (1). Since it has been reported (2, 3) that glucagon administration also has a protein catabolic effect in normal rats, it was decided to test the effects of this hormone on protein metabolism in pregnant rats to see if a resistance to its effects could be demonstrated.

A pregnant and a nonpregnant group of rats with an initial average body weight of 212 gm. were injected subcutaneously with 0.25 mg. of glucagon[2] in water twice daily (0.50 mg./day) from the 13th to 20th days of gestation. Similar control groups were injected with water and pair fed with the glucagon treated rats. Average food intakes in gm./rat/day were as follows: pregnant = 7.7, nonpregnant = 7.7. The diet and experimental methods were as described previously (1). All animals were killed by stunning and decapitation on the 21st day of gestation after an 18 hour fast. The experimental results are shown in Table 1. Statistical analyses were carried out by means of the "t"

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[2] Glucagon, lot 400–2, supplied by Dr. A. M. Fisher, Connaught Medical Research Laboratories, University of Toronto.

test and the significance of the difference between means is indicated by the value of "P".

The experimental results clearly indicate that pregnant rats exhibit no more resistance to the effects of glucagon than do nonpregnant rats. Significant losses of body weight were produced by the hormone in both pregnant and nonpregnant rats. The activities of the alanine-glutamic and aspartic-glutamic transaminases were increased to a similar extent in pregnant and nonpregnant rats. Other effects of the hormone were a decrease in blood amino nitrogen levels and an increase in liver weight. Blood urea levels and packed cell volumes were not significantly affected by the treatment.

Glucagon has been reported to cause an elevation of blood sugar. It has been shown that this effect is only transient and that it is much more clearly demonstrated in un-

TABLE 1. EFFECTS OF GLUCAGON ADMINISTRATION
(mean ± standard error)

	Nonpregnant			Pregnant		
	Glucagon	Water	Value of P	Glucagon	Water	Value of P
Body weight change during treatment (gm.)	− 20 ± 4	− 4 ±	< .01	− 6 ± 3	+ 26 ± 4	< .01
Liver weight (% body weight)	3.54 ± .16	2.91 ± .18	< .02	2.93 ± .14	2.66 ± .11	—
Liver alanine-glutamic transaminase activity[1]	87 ± 6	50 ± 7	< .01	61 ± 4	26 ± 1	< .01
Liver aspartic-glutamic transaminase activity[1]	204 ± 5	129 ± 4	< .01	198 ± 7	134 ± 2	< .01
Blood amino nitrogen (mg.%)	9.7 ± .3	12.2 ± .5	< .01	9.7 ± .6	11.9 ± .5	< .05
Blood sugar (mg.%)	62 ± 2	71 ± 3	< .01	48 ± 3	49 ± 3	—
Blood urea (mg.%)	27.0 ± 1.2	25.8 ± 2.2	—	26.1 ± .9	26.2 ± 1.4	—
Packed cell volume (%)	46.5 ± .7	46.8 ± 1.8	—	44.2 ± 1.7	40.0 ± 1.3	—
Number of rats	12	8		5	10	

[1] Transaminase activities are expressed as the μl. of pyruvate CO_2 formed per mg. wet tissue per hour.

fasted animals (4). In the present study, a significant lowering of blood sugar was produced by the hormone; however, these animals were killed in the fasted state 18 hours after the last hormone injection.

The adverse effect of glucagon on the course of gestation was very pronounced. At autopsy, it was found that the viable fetus weight was greatly reduced by the administration of the hormone (control = 4.23 ± .17 gm., glucagon = 3.02 ± .32 gm., $P < .01$). A great many resorptions and macerated fetuses were found in the glucagon-treated group; none were found in the control group. These effects were even more pronounced in another study employing a dosage of 1 mg./day. Although the administration of large doses of cortisone significantly reduces the fetus weight, it does not cause resorption or maceration of the fetuses in rats as does glucagon.

Some of the effects of glucagon administration in nonpregnant rats are quite similar to those of cortisone treatment. Both hormones lead to a decreased food intake. Elevated alanine-glutamic transaminase activity and liver weight and decreased blood amino nitrogen levels and body weight gains are characteristic of treatment with either compound. Beaton *et al.* (5) have associated an increase in alanine-glutamic transaminase activity with an increase in protein catabolism; the present findings are in agreement with this concept. One striking effect of glucagon not seen with cortisone is the pronounced elevation of aspartic-glutamic transaminase activity; cortisone has no effect on the activity of this enzyme.

It appears that although pregnancy affords a protection against the biochemical effects of cortisone administration, it gives no protection against the effects of glucagon.

The resistance of the pregnant rat to the effects of cortisone administration is not part of a general resistance to all protein-catabolic compounds.

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EFFECTS OF DESTRUCTION OF THE THORACIC SPINAL CORD ON SPERMATOGENESIS IN THE MOUSE[1]

ABSTRACT

Ten out of eleven male mice with proven ultrasonic obliteration of the 8th to 10th thoracic cord segments, for as long as two months, showed no significant differences from control mice with respect to testicular weight, morphology and spermatogenesis. The significance of these findings is discussed.

The following report deals with a study of localized spinal cord destruction and its effects on spermatognesis in the mouse; to our knowledge, the first such study performed on animals. Specificity of lesion location and absence of surgical trauma have been attained by the recently developed technic of focused ultrasound (1).

MATERIALS AND METHODS

Focused ultrasound was applied to the shaved skin overlying the 8th to 10th thoracic spinal cord segments of 56 male albino mice, three months of age. Fifteen to thirty doses of ultrasound were administered (frequency, 2.5 millicycles per second; intensity, 900 volts; pulse duration, 0.6 sec.; pulse period, 1.0 sec.).

A regimen of constant room temperature, manual feedings with water-softened Purina Laboratory Chow cubes and water by eye-dropper, as well as two daily manual expressions of the urinary bladder, was carried out for the first two weeks. After this period, the mice required little special care. Untreated control and lesioned mice of the same age were sacrificed at the following times: 4 control and 4 lesioned mice at one week following ultrasound treatment; 4 each at one month; and 5 control and 3 lesioned mice at two months. Body and testicular weights were taken at sacrifice. The testes, epididymis, urinary bladder, kidney, lung and spinal cord were fixed in 10% formalin or in Orth's fluid; dehydrated; embedded in paraffin; sectioned and stained. The cords were stained for myelinated nerve fibers after Lillie's modification of the Weil-Weigert method (2). The other tissues were stained with hematoxylin and eosin.

RESULTS

Eleven of the lesioned mice showed immediate paraplegia and remained in this state until sacrifice. The remaining animals died from a contagious hemorrhagic pneumonitis. The eleven experimental mice discussed in this report showed no damage of skin through which the ultrasound was focused; but possessed completely scarred spinal cords containing no stainable myelinated fibers. One exception was a mouse killed two months after treatment, in which a few peripheral patches of myelinated fibers remained.

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There was no significant difference in testicular weight, between experimental and control mice, with the exception of one mouse killed at one month after treatment in which the testes weighed only 34% of the control values.

Sections of testes, taken from various regions, showed no histological differences between control and experimental mice with respect to seminiferous tubule diameter, number of intratubular cells, per cent of intratubular cells which were Sertoli cells, or spermatozoa, with one exception: in the mouse killed at one month after treatment the testes showed a reduction in tubular lumen diameter and total number of intratubular cells, very few spermatozoa, and a relative increase in percentage of Sertoli cells, as compared to control testes.

Lesions in the remaining tissues were limited to gross urinary bladder distention in the mice one week following ultrasound treatment.

DISCUSSION

This experiment demonstrates that the integrity of the spinal cord is not needed for spermatogenesis in the mouse. The results of this study contradict the theory of Krücke (3) and Gaupp (4), which maintains that the myelinated *fasciculus parependymalis*, allegedly extending from hypothalamic connections in the medulla to cauda equina directly controls sperm formation in animals and man. The phenomenon of partial or total spermatogenic arrest, shown by testicular biopsy or seminal sperm counts in a high percentage of human traumatic paraplegics (5–7), cannot therefore be explained by this theory.

That peripheral nerves coursing to the testes are not needed for spermatogenesis has already been suggested by the work of Berthold (8) who found sperm formation in abdominally implanted testes in cocks. Such findings have been confirmed more recently in mice (9). Disruption of dorso-lumbar sympathetic nervous pathways, however, results in a temporary decrease or absence of spermatogenesis in cats (10–11), guinea pigs (12), and perhaps in man (13–16). In this study, however, a depression of spermatogenesis did not occur *at any time* after ultrasonic treatment, although the lesions (T.8–T.10) extended to the upper limits (T.6–T.10, according to Kuntz (17)) of sympathetic nervous outflow from the spinal cord to the vasculature of the male reproductive tract. If, in some manner, disruption of these sympathetic nervous pathways does depress fertility, it is conceivable that vasomotor reflex centers in the mouse cord below the level of the lesions were spared in this study; thus permitting the vascular tone which would be temporarily destroyed by dorso-lumbar sympathectomy to be maintained. The existence of such reflex centers has been suggested by work on dogs and man (18, 19). Consequently, the high segmental level of lesions in our mice does not exclude the possibility that some sympathetic nervous influence on spermatogenesis exists.

Why one of the eleven experimental mice showed testicular atrophy and decreased spermatogenesis, could not be explained.

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PRE-ALBUMIN: A THYROXINE-BINDING PROTEIN OF HUMAN PLASMA

ABSTRACT

During electrophoresis in Tris-maleate buffer, pH 8.6, I^{131}-labelled thyroxine added to serum migrates in association with moieties both in the inter-alpha zone and in a zone approximately 20% more anodal than albumin. This finding is in contradistinction to those obtained during electrophoresis of serum in veronal buffer, wherein I^{131}-thyroxine migrates principally in the inter-alpha zone (thyroxine-binding protein, TBP).

Electrophoresis in Tris-maleate makes possible assessment of the absolute thyroxine-binding capacities of both TBP and pre-albumin. In normal serum, the binding capacity of TBP is approximately 25 μg./100 ml. and that of pre-albumin is approximately four times as great.

Several properties are described which suggest that TBP and pre-albumin are distinct entities.

In 1952, Gordon, Gross, O'Connor, and Pitt-Rivers first described a characteristic migration of thyroxine in serum during electrophoresis in filter paper, using barbital buffer at pH 8.6 (1). Both endogenously and exogenously labelled hormone were found to migrate in association with moieties in the zone between the α_1 and α_2 globulins. The protein carriers with which thyroxine was presumed to have been associated during electrophoresis have since come to be known as the thyroxine-binding protein of plasma (TBP). Much effort has been directed toward elucidation of some of the physicochemical and physiological properties of TBP (2–5). It was soon learned that although the major

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portion of tracer concentrations of labelled thyroxine were associated with TBP, progressively larger fractions of labelled thyroxine became associated with albumin as the concentration of stable thyroxine in the serum was increased. Thus, TBP and albumin were assumed to be the primary and secondary carriers of thyroxine, respectively. Determination of the partition of labelled thyroxine between TBP and albumin in serum containing varying quantities of stable thyroxine has been employed as a means of assessing the relative thyroxine-binding activity of TBP in normal and abnormal sera (6). By means of this technique, alterations in TBP activity have been found in pregnant and aborting women (6). However, efforts to quantify the absolute thyroxine-binding capacity of TBP using conventional electrophoresis have failed, since calculated values for the total quantity of thyroxine bound to TBP continued to increase as the concentration of stable thyroxine was augmented. This failure to achieve plateau values was ascribed to trailing by albumin across the TBP area during electrophoresis (4). For this reason, Robbins devised a system of reverse-flow zone electrophoresis in which plateau values for the total quantity of thyroxine bound to TBP could be obtained. By means of this technique, the thyroxine-binding capacity of TBP in normal serum has been estimated to be approximately 20 μg./100 ml. (7).

Recently, an electrophoretically and ultracentrifugally homogeneous preparation of a highly active, inter-alpha-migrating TBP has been obtained (8). Preparative methods, as well as several of the properties of the purified TBP will be described in a later communication. However, during the isolation of fractions of human plasma proteins progressively enriched in TBP, another highly potent thyroxine-binding protein became manifest. This protein could be demonstrated during electrophoresis of fractions in barbital buffer at pH 8.6, wherein it displayed a characteristic migration, moving toward the anode approximately 20% faster than serum albumin. This "pre-albumin" has also been isolated in apparently pure form, and has been found to be exceedingly avid for thyroxine (8).

Binding of thyroxine by proteins migrating ahead of albumin during electrophoresis in barbital buffer had already been noted in cerebrospinal fluid and in the serum of normal and nephrotic patients (9, 10), but its significance was not understood.

In the light of the foregoing observations, it became of interest to assess the possible role of pre-albumin in binding thyroxine in whole serum. During the isolation of TBP, it had been noted that when serum containing labelled thyroxine was electrophoresed in a cellulose column (11), using Tris-maleate buffer,[1] pH 8.6, $r/2 = 0.2$, binding of thyroxine by moieties migrating ahead of the albumin could regularly be demonstrated. Tris-maleate buffer has therefore been employed during paper electrophoresis of sera containing labelled thyroxine. In this system, binding of thyroxine by pre-albumin is consistently demonstrable in normal serum (Fig. 1). When serum containing labelled thyroxine is supplemented with increasing amounts of stable thyroxine and electrophoresed in this Tris-maleate system, several findings of interest emerge. First, at very low concentrations of added thyroxine (0.4 μg./100 ml.), the percentage of labelled thyroxine associated with pre-albumin is as great or nearly as great as that associated with TBP. Second, absolute values for the thyroxine-binding capacity of both TBP and pre-albumin can be obtained without the use of the reverse-flow system. In confirmation of the findings in the latter system, saturation of TBP is achieved when the protein binds approximately 25 μg./100 ml. of normal serum. Pre-albumin, on the other hand, appears to have a thyroxine-binding capacity of approximately 100 μg./100 ml. of normal serum.

In contrast to labelled thyroxine, triiodothyronine, when added to serum and electrophoresed in the Tris-maleate buffer, displays essentially no binding to pre-albumin

[1] Use of the Tris-maleate buffer was suggested by Dr. R. R. Porter, National Institute for Medical Research, London, England.

(Fig. 1). However, its electrophoretic migration in this system differs from that in barbital. In barbital buffer, triiodothyronine binds in part to TBP, but the remainder trails backward toward the origin (12). In Tris-maleate, discrete localization of labelled triiodothyronine in the TBP and albumin zones is seen, and labelled triiodothyronine is progressively displaced from TBP to albumin as the concentration of stable hormone is increased.

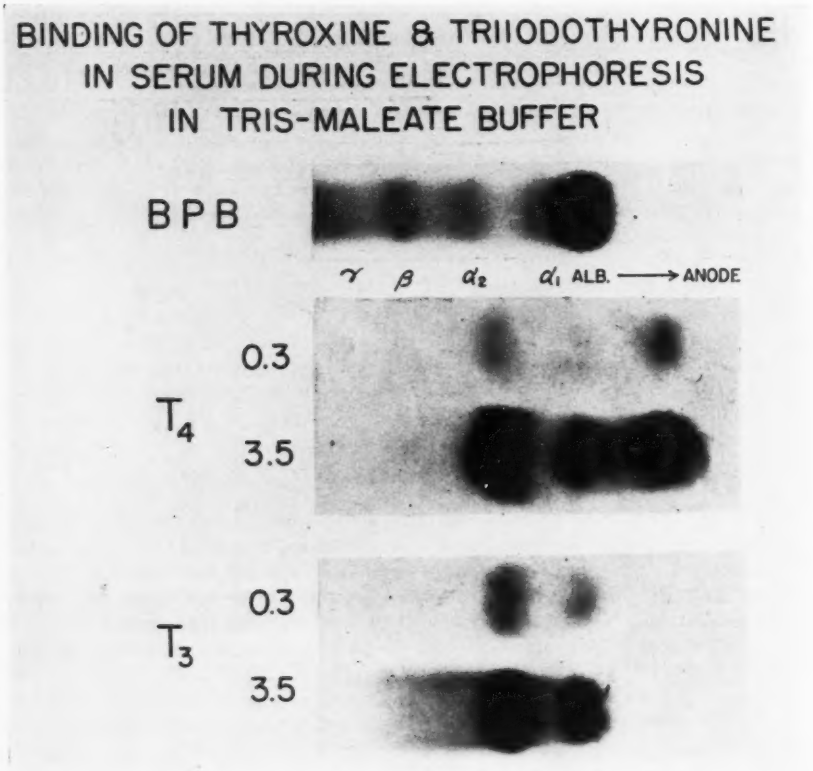

FIG. 1. Electrophoresis of normal human serum containing I^{131}-labelled thyroxine (T_4) and triiodothyronine (T_3), employing tris-maleate buffer, pH 8.6, r/2 = 0.20. BPB = brom-phenol blue stain of electrophoretic strips, revealing localization of protein. T_4 and T_3 = radioautographs of electrophoresed sera containing 0.3 and 3.5 μg. per cent of added hormone.

Pre-albumin seems distinct from TBP for the following reasons: 1) TBP is present in Cohn Fractions IV-7 and IV-8 of the serum proteins, but pre-albumin is not, 2) the individual proteins have been separately isolated, 3) the isoelectric point of TBP, but not of pre-albumin is altered by exposure to bacterial neuraminidase, 4) TBP binds triiodothyronine, but pre-albumin does not, 5) the absolute thyroxine-binding capacity of TBP in the Tris-maleate system does not differ from that found in the reverse-zone system, 6) in such states as pregnancy and untreated thyrotoxicosis, the thyroxine-binding capa-

ties of TBP and of pre-albumin vary independently. These findings, together with the occasional demonstration of binding of thyroxine by pre-albumin in a variety of biological fluids electrophoresed in barbital buffer suggest that the pre-albumin is not an artefact induced by the Tris-maleate system, but rather that in barbital buffer its presence is obscured by either trailing or protein interactions. The physiological role of pre-albumin, as well as its thyroxine-binding capacity in a variety of abnormal states, is now under investigation.

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[2] A portion of the work herein described was done during tenure as Visiting Worker, National Institute for Medical Research, London, England, 1956–1957.

THE IMPACT OF BIOCHEMISTRY ON ENDOCRINOLOGY

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Fellow Members of the Endocrine Society and Guests:

IN MY brief tenure as an officer of the Endocrine Society, I have often heard complaints that the clinician was being crowded from the annual programs. On the other hand, I have had the unusual experience of having clinicians complain of the time given to clinical symposia. This led me to study what had really happened to this Society over the years. I tabulated the professional degrees by which the authors and coauthors of the first fifty papers in this year's program were designated. The ratio of M.D.s to Ph.D.s was 77%:23%. The distribution 20 years ago when the Society also met here was almost identically the same: 76% for M.D.s and 24% for Ph.D.s

What then had led to the remarks? Was it simply a failing memory of the past, or had some change taken place in the meaning of the labels? As my wife says, ten years ago the initials IBM would have meant only one thing: International Business Machines; now if one says IBM to the younger generation its members immediately think of Intercontinental Ballistic Missile.

A similar change seems to have occurred in the meaning of M.D. The days when descriptive case reports were presented by the up-and-coming internist are gone; today the approach is physiological and biochemical. This led me to the topic I have chosen for this evening, "The Impact of Biochemistry on Endocrinology."

I do not claim to be an unprejudiced observer; in fact, I do not think that anyone can be objective about a subject in which he is vitally interested. You may have heard of one way to identify a musician: if he hears a strange woman singing in the bathroom he puts his ear to the keyhole. I hope, however, that the broad sweep of past, present and future will iron out my petty prejudices. I am going to say tonight that biochemistry was responsible for the conversion of endocrinology from theory to science, that the future of endocrinology will be more and more intimately tied up with biochemistry, and that while this is inevitable, it is by no means an unmixed blessing.

The concept of an endocrine system is an anatomico-biochemical one. It came late in the development of medicine because, unlike other structural systems, it could not be conceived by the eye alone. The nerves ran from the brain and spinal cord to muscles and other organs. The muscles connected two parts of the body, and when the stimulus was applied to a nerve leading to a muscle, the latter would swell and shorten, drawing the connected parts together. The blood vessels led from the heart through

progressively smaller branches to all the tissues, and the flow of blood could be observed when the heart contracted. Structures like the pancreas and the salivary glands had duct systems through which fluid could be demonstrated to flow when the individual was placed under certain conditions or specific nerves were stimulated. The course of the ovum through the fallopian tube to the uterus, and the progress of the sperm through the vas deferens could be traced, but this did not explain why the mammary glands regressed when the ovaries were removed or why a man's beard would not grow in the absence of the testes. The idea that cells could form chemical compounds and could release them, the biochemical concept, was necessary before there could be a discipline such as endocrinology. Thus, endocrinology developed later than other medical fields.

The basic biochemical nature of endocrinology made it peculiarly susceptible to quackery during the physiological period of its development. One of the original methods of demonstrating the endocrine function of an organ was to transplant the organ to another region of the body of an experimental animal, or to another animal; if the condition produced by its removal was relieved by the transplant, the effect was hormonal and the gland had an endocrine function. Since immunology was also in an elementary state of development, it did not seem illogical to attempt relief of human deficiencies by the transplantation of human or mammalian tissues. Because this was a profitable application, many were willing to accept logic against data. How I remember with a certain nostalgia, the days of Brinckley and his goat glands!

Then too, since the hormone of the thyroid gland was active by mouth, it was easy to make the broad generalization that all hormones would be active orally, and again those more interested in logic than in experiment brought opprobrium on endocrinology by continuing to apply this general concept long after experiments had proved its uselessness in most cases. It was only as biochemical techniques began to be applied to endocrine problems in the 1920s, beginning with the isolation of insulin, that endocrinology began to move from the twilight land of medical mumbo-jumbo into its rightful place in the sun.

Now how has biochemistry contributed to the conversion of endocrinology into a respected medical specialty? The biochemical epoch in endocrinology can be divided roughly into three periods which tend to overlap. The first is the period of isolation and structural identification, which began with the crystallization of epinephrine by Takamine and Aldrich. Work on the protein hormones has now reached this stage.

The second period is the quantitative and dynamic phase. It is converting endocrinology into a more exact science today, a field in which the individual components and their changes can be quantitatively measured.

The third period is in its infancy. During this era the molecular mechanisms of hormonal control will be understood. Although the results on human life are veiled in the mists of the future, I predict that this knowl-

edge will lead to far greater human control of biological processes.

What has the first biochemical phase contributed to endocrine thinking? While epinephrine was the first hormone the structure of which was established, the absence of the adrenal medulla did not produce the profound changes in the organism associated with the other endocrine tissues. As Cannon said, it seemed to function more as an "emergency hormone" and interest centered in its pharmacological action rather than in its hormonal function. The discovery of insulin aroused new interest. The crystalline product was obviously a protein which was destroyed in the intestinal tract, not all endocrine difficulties could, therefore, be relieved by oral administration. It was the excellent chemical work of Butenandt, Doisy and others at the end of the 20's, however, showing that the sex hormones were structures related to cholesterol, which broadened the thinking of endocrinologists beyond proteins and amino acid derivatives, and opened a new field of organic chemistry that still occupies the energies of many capable chemists. Now the biochemistry of the steroid hormones, like that of the thyroid, has largely passed into the second phase, but during the first phase of its development it laid low the fantasy that all hormones are water-soluble, and that one could expect to solve all endocrine problems by the administration of aqueous extracts.

With the development of simple chromatographic micromethods for the quantitative determination of the individual amino acids, and for the separation of peptides, the protein hormones also entered the period of isolation and structure determination, with implications for the future of both biochemistry and endocrinology even more profund than the discovery of the steroid hormones. Proteins are so complicated chemically that fifteen years ago most biochemists would have said that to expect exact knowledge of the detailed structure of a protein molecule within fifty years was an optimistic estimate. But in the meantime paper chromatography developed. With that combination of ingenuity and perseverance which constitutes genius, Sanger took advantage of this technique, together with a knowledge of certain organic reactions and a gift for logical sequence which others waste on solving crossword puzzles, to work out the structure of insulin. Today we can write its formula with the same precision as that of cortisol. These techniques, further improved, enabled Du Vigneaud to determine the structure of oxytocin and vasopressin; and while these polypeptides were more simple than insulin, their organic synthesis by Du Vigneaud was another landmark in chemistry.

Today the detailed structures of six protein or polypeptide hormones are known and much has been learned of the amino acid sequences of several others. Species differences in the composition of a number of these hormones have been identified; in the case of the growth hormone these are sufficiently great to explain why the pure cattle and sheep hormones are ineffective in man or primates. On the other hand, surprising similarities have been found between sequences in different hormones from the same species, as in oxytocin and vasopressin, and in ACTH and melano-

cortex stimulating hormone. These have explained apparent overlapping effects, such as bronzing in the Addisonian and in the patient treated with ACTH. Experience with ACTH indicates that portions of the original molecule may be adequate for activity; this holds out the hope that species differences in the structure of a hormone may not necessarily be part of the active portion, and that active species-nonspecific peptide molecules can be prepared.

As we mentioned earlier, the steroid hormones, the thyroid hormones, and in a minor way epinephrine, have entered the second period of biochemical development, the quantitative and dynamic phase. The effects on endocrinology have already been widespread, both in basic understanding and in diagnosis and treatment, with still greater implications for the future. The development of quantitative microchemical colorimetric methods with considerable group specificity, and the coming of chromatography with its remarkable ability to separate compounds of similar structure made possible over-all studies of levels and rates; the use of radioactive isotopes permitted the measurement of sequential processes.

Wilhelm Zimmermann's discovery of the metadinitrobenzene reaction for 17-ketosteroids ushered in this period. Coupled with the studies of Callow and of Dorfman and coworkers, which demonstrated the major metabolic products of the neutral steroids, the applications of the Zimmermann method and its modifications enabled us to recognize the variations of steroid endocrine function with age and sex, and to identify the contributions of the adrenal glands to androgen levels. Over the years it has been a valuable diagnostic tool and an antidote to quackery.

In the late thirties Treverrow showed that thyroxine was carried in the blood combined by secondary binding forces with plasma proteins, and micromethods were developed for the determination of protein-bound organic iodine. This was the forerunner of other studies that demonstrated that the plasma proteins have a great influence on the diffusibility of hormones from the circulation. The determination of protein-bound iodine also distinguished thyroid function from other influences on basal metabolic rate, and the lack of correlation of the two values in pregnancy led to still more important discoveries regarding plasma proteins and hormones.

During the 1940s the various reducing reactions and the phenylhydrazine reaction of Porter and Silber, applied to urine extracts, broadened our understanding of adrenal function and improved diagnosis. Finally, Nelson combined chromatography and the Porter-Silber reaction to introduce the first feasible method for the quantitative determination of a steroid hormone in blood. This made possible the estimation of the levels of cortisol in which the tissues were bathed. By taking a series of samples after the injection of a standard dose of the hormone, a measure of the rate of its metabolism could be obtained, as well as the extent of its distribution; and by making similar measurements after the injection of a standard amount of ACTH, the ability of the human adrenal to respond to the pitui-

tary stimulus could be evaluated. Thus, much was learned of the dynamics of the pituitary-adrenal system in the human being.

The greater delicacy of paper chromatography, combined with sensitive color reactions, permitted both more detailed study of blood steroids of adrenal origin, revealing species differences, and the measurement of new steroids in blood, such as dehydroepiandrosterone, progesterone and androsterone. While these methods have not yet achieved routine use, they have thrown new light on steroid metabolism in the human subject, and may well have diagnostic value. For instance, it would appear that interstitial cell capacity can be determined by measurement of the changes in androsterone levels after injection of a standard dose of chorionic gonadotropin, much as adrenal capacity can be tested by the ACTH infusion test. Not only this, but methods for other steroids in blood are under development. We can reasonably look forward to the time when we can assess the concentrations of steroid hormones of all types, save perhaps the estrogens, on one 25 ml. specimen of blood, and can evaluate the function of the glands producing them by analysis of a series of such samples.

Combined with paper chromatography, the use of radioactive isotopes has opened up a storehouse of understanding of the biosynthesis, transport, and metabolism of the hormones. Using radioiodide, the biochemistry of thyroid function has been worked out. The trapping of iodide and the sequence of reactions by which thyroxine is formed have been elucidated quantitatively as well as qualitatively by our medalist of this evening, Professor Chaikoff, and by others. In the process we have come to understand the mechanisms by which the various antithyroid drugs act, and therefore can use them more effectively. The structures of the secreted hormones could also be determined, and their rates of penetration into tissues could be examined. Today the measurement of thyroid dynamics is a routine diagnostic procedure.

The application of an offshoot of paper chromatography, paper electrophoresis, to the plasma proteins of individuals having labeled thyroid hormones circulating in their blood has introduced a new concept of hormonal control by regulation of hormone distribution. As this audience knows, at normal hormone levels the major portion of circulating thyroxine is strongly bound by small amounts of a specific protein. At high levels the strongly binding protein is saturated and more of the hormone is attached to plasma albumin by weaker forces. Thus the amount which could readily diffuse into tissues is increased disproportionately to an increase in concentration of plasma hormone. The high levels of protein-bound thyroid hormone in pregnancy associated with normal basal metabolic rates have been shown to be due to an increase in the concentration of the strongly binding specific protein.

The same type of situation has now been demonstrated with cortisol. The protein involved in the strong binding of cortisol appears not to be the same as that which binds thyroxine; thus, the variation in concentra-

tion of specific binding proteins may come to play an important part in endocrine medicine, since it would determine the availability of the circulating hormones. This may be a general mechanism of regulating hormone transport, and alterations of specific plasma proteins could cause disease.

As already mentioned, the demonstration of biosynthetic sequences in the thyroid gland has been fruitful in elucidating different types of thyroid abnormality, and in explaining the action of different anti-thyroid drugs. This concept of biosynthetic order has also been valuable in the steroid field. Hechter and coworkers at the Worcester Foundation studied the products formed when various steroids were perfused through adrenal glands. From their results they postulated a certain sequential "peck order" in the various oxygenations. Various workers have studied the individual enzyme systems in acellular preparations and the results have borne out the original hypothesis as the preferred, although not obligatory, order of reactions.

In the meantime, Wilkins, knowing that the levels of the usual metabolites of cortisol were low in the urine of patients suffering with adrenogenital syndrome, and that the injection of ACTH increased the already high excretion of 17-ketosteroids, attempted treatment by suppressing ACTH secretion with cortisol. The spectacularly successful results are known to everyone in this room.

From the rather gross analyses of urinary steroids then available, Wilkins correctly interpreted this condition as an abnormality of steroid biosynthesis. Jailer postulated that this was an enzymic block to the further hydroxylation of 17α-hydroxyprogesterone on the basis of the increase in urinary 17-ketosteroids after ACTH administration, and the identification of pregnanetriol in such urine by Butler and Marrian. By the identification of certain 21 carbon steroids with paper chromatographic techniques Bongiovanni has now been able to demonstrate two different enzymic deficiencies in the biosynthetic sequence of Hechter, either of which lead to this syndrome. Thus this condition joins the growing number of human ailments which can be explained as abnormalities of specific enzymes.

We could not speak so glibly of treating these patients with cortisol, or of adrenalectomy and hypophysectomy for mammary cancer, if it were not for some fundamental discoveries of the microbiological biochemists. Most bacteria do not contain measurable amounts of steroids. Yet it was found that certain strains could be trained to carry out specific oxidations on steroid molecules. Bacterial oxidations are the basis of most industrial preparations of active steroids today, and make their use in medicine economically possible. These findings have also contributed to the general concept of the plasticity of enzyme synthesizing systems.

Another concept with important significance in understanding disease is that of the qualitative similarity of the biosynthetic systems in all tissues forming steroid hormones. It was found that the first steps in the sequence

outlined by Hechter for the adrenals also occurred in the testicular interstitial cells, but a reaction which was normally low in the adrenal, the splitting off of the two carbon side chain from 17-hydroxyprogesterone, was a major activity of these cells. In the original work, however, attention was called to the presence of polar 21 carbon steroids in the products of the incubations and it was hypothesized that further hydroxylations similar to those in the adrenals might also occur to a small extent. This has since been confirmed. In the meantime others had demonstrated that the ovaries could carry out a similar sequence of reactions and could, in addition, aromatize ring A of either testosterone or androstenedione. The adrenal also was shown to be capable of the latter conversion. Thus the concept has emerged that these tissues, all of which arise in the region of the genital ridge in the embryo, have the same set of biosynthetic enzymes, but differ in the relative concentrations of individual members.

Both congenital hyperplasias and malignancies of any one of these organs have long been shown to simulate the hormonal action of another. This would not appear to involve "embryonic cell rests" of the other tissue, as the older pathologists thought, or the *de novo* formation of new enzymes, but simply an abnormal alteration in the rate of reproduction of certain enzymes normally present in small amount. Thus, biochemical investigation has had far-reaching effects on concepts in endocrinology.

INFLUENCE OF ENDOCRINOLOGY ON BIOCHEMISTRY

We have been looking at the impact of biochemistry on the development of endocrinology as a science and a medical discipline, but I would like to call attention briefly to the contributions which endocrinology has made to biochemistry.

First, the discovery that such important compounds as the sex hormones and adrenal hormones were related to cholesterol in structure, was a great impetus to the study of steroid biochemistry. Many plant and animal steroids would not be known, let alone their structures determined, if it were not that they could conceivably serve as industrial precursors of the steroid hormones. Many techniques in microchemistry and in paper chromatography were developed during the study of steroid hormone biochemistry.

Second, protein chemistry owes much to endocrinology. The importance of insulin in the treatment of diabetes made its preparation an industrial process, and both its availability and importance kept it always in the forefront of protein research. Today the methods developed by Sanger for insulin are being applied to enzymes to understand their structure and mechanism of action, the accomplishments of Du Vigneaud in synthesizing the posterior lobe hormones have spurred attempts at other syntheses, and the recognition of species differences in the structure of proteins performing the same function has affected thinking in regard to all active proteins.

Third, the desire to explain the mechanisms of action of hormones like insulin, epinephrine and thyroid hormone has led to greater knowledge of

energy metabolism and lipid synthesis, even though the basic hormonal mechanisms have not yet been found.

Fourth, the probability of the transmission of antidiuretic hormone along nerve fibers and its apparent synthesis in nerve cells, as well as similar hormonal mechanisms in insects, has led to new views of the biochemistry of the nervous system which may have far-reaching effects. Other examples could be cited. Thus, the attempts to solve the problems of one type of biological phenomena have had repercussions on many others.

THE FUTURE

What then of the future? In my prejudiced view I can only see a more intimate relationship between biochemistry and endocrinology. The knowledge of protein hormones is now at the stage where understanding of the steroid hormones was 20 years ago. I think we can reasonably expect that the future course will be similar. Ways will be found to modify structures to get around species barriers; perhaps reactive portions of the natural structures will be found and these will be synthesized. Microchemical methods will be developed for measurement of blood levels as soon as distinctive groups are identified in the molecules.

Knowledge of the metabolic enzymic reactions involved in the biosynthesis of the hormones offers the opportunity to look for specific inhibitors. We already have such compounds in the antithyroid drugs and amphenone; I think that there is a reasonable chance that we shall find such compounds for every major biosynthetic reaction. Thus we shall be able to control excessive or abnormal production of hormones.

The development of more and better methods for determination of the hormones and their metabolic products in body fluids will lead to greater precision in diagnosis.

Study of the alterations in biosynthetic systems during development of the malignant change in endocrine tissues could lead both to a better understanding of the causes of malignancy and to better means of interfering with such growths once they develop.

Thus far, we cannot, with certainty, state in chemical terms how any hormone acts on a target tissue, although epinephrine and estrogens have been associated with specific enzymes. I predict that there will be a major breakthrough in this area before long, however, and that thereafter we shall rapidly unravel the various mechanisms by which enzymic reactions are hormonally regulated. Once this is found out, we shall soon develop methods of control based on this knowledge.

There is a good possibility that we shall discover other hormones. If we accept Darwinian evolution, new reactions in living matter arise by mutation, an accidental discontinuous process; their survival depends on their value to the whole animal in its environment, a sorting which is always going on. In other words, while the most efficient method of accomplishing a given process will not necessarily have been tried, little that is useless to the organism will have survived. I think, therefore, that the

reason we have not found a specific function of some organ like the pineal gland is that we haven't looked with the right tools. Most of the hormones which we know have been discovered by gross anatomical and physiochanges. As our methods of analytical study become more precise, not only shall we know more about the mechanisms by which the present hormones act, but we shall discover new controlling systems of hormonal type. We shall also come to understand better the interplay between the nervous system and the endocrine system at a molecular level, and thus shall find more direct means to control this interaction.

What is the significance of all this for medicine? It means that the specialist in endocrine medicine will become more and more important, as the means of control of hormonal action become more multiform. But it also means that such a specialist must understand the biochemical background of endocrine problems. Moreover, he will be increasingly dependent on the biochemical laboratory for diagnosis and for monitoring treatment. Medicine will remain an art when it comes to dealing with the patient as an individual, but it will be a science when it comes to the results which can be achieved.

I think these developments, barring an atomic war which will take us back to the dark ages, are inevitable. "Wonderful" you will say, "many of the misfortunes of life will be reduced or eliminated." But now I claim my privilege to "view with alarm." Where is all this leading us?

First, it inevitably means greater centralization in medical treatment, for more specialized equipment and personnel will be required. Further, it will be expensive, for it will require a significant amount of time from individuals who must be both intelligent and well-trained. This, as part of the over-all development of medicine, means that it will be centrally financed, either through insurance or government medicine, and more easily subject to central regulation.

Second, greater control of endocrine function will not only lead to regulation of population, a goal already within sight, but also to influence over emotional reactions, since they are conditioned so much by endocrine factors. There will be greater human uniformity. As a Swedish scientist said to me one time when we were discussing Swedish politics, "We Swedes are so much alike that all a politician needs to do to know how a given piece of legislation will be received is to go out and see what ten other Swedes think about it. He'll have a fair idea of what the reaction of the nation will be." While this makes for greater stability, it also means that there can be greater control over the reactions of the people.

Finally, in the long view I see that the intimate knowledge of the biochemistry of endocrinology may lead to a civilization resembling a human bee colony, efficient in serving the needs of its members, and particularly the queen, but made up of individuals so well adapted that they would be exceedingly dull to an inhabitant of this room reincarnated in the twenty-third century.

THE ENDOCRINE SOCIETY

1959 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional

promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than September 15th each year.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by September 15th each year.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.